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Úloha NK buněk v patogenezi autoimunitní artritidy

NK cell involvement in the pathogenesis of autoimmune arthritis

Disertační práce

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V Praze, 15. 12. 2014

Mgr. Jan Richter

Děkuji své školitelce MUDr. Anně Fišerové, CSc. za odborné vedení, zajištění výborných pracovních podmínek a profesionální podporu v celém průběhu svého studia. Dále děkuji kolegům z imunologické skupiny laboratoře Molekulární biologie a imunologie Mikrobiologického ústavu AV ČR, v.v.i. za veškerou odbornou pomoc. V neposlední řadě děkuji své manželce a celé rodině za veškerou podporu a pomoc.

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Abstrakt

Revmatoidní artritida, jakožto jedno z nejčetnějších autoimunitních onemocnění vůbec, představuje celosvětový problém. Navzdory častému výskytu této choroby nebyla její patogeneze dosud zcela objasněna. Revmatického procesu se účastní řada imunitních buněk od antigen-prezentujících buněk po T, B a NK buňky. V této práci jsme se zaměřili na NK buňky exprimující repertoár aktivačních a inhibičních receptorů, které ovlivňují jejich funkci ve zdraví i nemoci. Zaměřili jsme se na analýzu funkce NK buněk a popsali možnosti její modulace revmatickými autoantigeny a multivalentními glykodendrimery nesoucími 4 (GN4C) nebo 8 (GN8P) N-acetyl-glukosaminových skupin. Jejich efekt na NK buňky a glykosylační dráhy byl studován *in vitro*. *In vivo* studie provedená na kolagenem-indukované artritidě (CIA) – myším modelu RA – byla provedena ke zjištění efektu těchto látek na rozvoj klinických příznaků choroby a vybrané imunitní parametry.

Srovnání NK-buněčné cytotoxicity u pacientů s RA, dalšími zánětlivými onemocněními a u zdravých dárců prokázalo její poškození především právě u pacientů s RA. NK buňky periferní krve reagovaly na glykokonjugát GN8P inhibicí své efektorové funkce u vzorků s vysokou expresí CD161. Exprese mRNA pro glykosyltransferázu MGAT5 byla zvýšena v synoviocytech pacientů, ale ne v periferní krvi, což naznačuje vliv aberantní glykosylace na autoimunitní procesy. Dále jsme zjistili, že NK buňky reagují na autoantigen MCV specifický pro RA zvýšenou expresí CD161, a to ve vzorcích krve zdravých dárců, ale ne v krvi pacientů s RA, kde byl tento parametr již zvýšen v důsledku probíhající nemoci. NK buňky jsou navíc schopny exprimovat citrulinační enzym PAD4, což ukazuje na jejich možnou roli v citrulinačních procesech vedoucích k produkci autoantigenů specifických pro RA.

Glykokonjugát GN4C byl použit v *in vitro* studii sledující expresi glykosyltransferáz v NK buňkách. Zjistili jsme, že toto glykomimetikum zasahuje do glykosylačních procesů snížením exprese jak MGAT3, tak MGAT5, což jsme prokázali v čerstvě izolovaných NK buňkách zdravých dárců, i v NK-buněčné linii NK-92. V obou modelech jsme pozorovali zvýšení cytotoxicity a stimulaci Th1 cytokinů TNF-α, IFN-γ a zejména IL-2 podporujících NK-buněčnou cytotoxickou aktivitu.

V myším modelu RA jsme potvrdili progresivní poškození NK-buněčné aktivity podobně jako u lidské RA, což odpovídalo i relativnímu zastoupení NK buněk. Zjistili jsme, že GN4C je účinnější ve smyslu zlepšení klinických příznaků, redukce zánětlivé synoviální infiltrace a útlumu aktivovaných antigen prezentujících buněk nezbytných pro aktivaci autoreaktivních T lymfocytů. Sledováním humorální imunitní odpovědi na podání glykodendrimerů jsme zjistili potlačení vzestupu IFN-γ a TNF-α a stimulaci IL-4, což koreluje s redukcí IgG2a specifického pro CII.

Tato práce přináší přehled zapojení NK buněk v RA a role glykanů v *in vitro* a *in vivo* podmínkách u pacientů s autoimunitou a zdravých jedinců. NK buňky reagují na autoantigeny specifické pro RA a účastní se citrulinačních procesů a následné progrese RA. Užitím multivalentních glykodendrimerů *in vivo* je nicméně možné inhibovat autoimunitní procesy a docílit významného potlačení klinických symptomů u myší, což naznačuje potenciál pro zefektivnění terapeutických přístupů v časných stadiích RA.

Abstract

Rheumatoid arthritis (RA) is a worldwide problem representing one of the most prevalent autoimmune diseases in the world. Despite the commonness of the disease, its pathogenesis has not been fully described. Immune cells ranging from antigen-presenting cells to T, B and NK cells playing various roles participate in the rheumatic process. In this work we concentrated on NK cells expressing a repertoire of activating and inhibitory receptors which influence their function in health and disease. We focused on the analysis of NK cell function and described its possible modulation by rheumatic autoantigens and multivalent glycodendrimers bearing 4 (GN4C) or 8 (GN8P) N-acetyl glucosamine moieties. The effect on NK cells and the glycosylation pathways was further studied *in vitro*. Finally, an *in vivo* study was performed on an animal model of RA – collagen-induced arthritis (CIA) to assess the effect of the compounds on clinical development of the disease and selected immune parameters.

Comparison of NK cell cytotoxicity in patients suffering from RA, other inflammatory diseases and healthy donors showed its impairment particularly in RA patients. Peripheral blood NK cells reacted to GN8P glycoconjugate by inhibition of their effector function in CD161 high-expressing samples. The MGAT5 glycosyltransferase mRNA expression was increased in RA patients' synoviocytes but not in peripheral blood, suggesting the involvement of aberrant glycosylation in the autoimmune process. We found out that NK cells react to RA-specific autoantigen (MCV) by increased expression of CD161 in healthy donor samples but not in those from RA patients where the levels had already been elevated due to the ongoing disease. Moreover, NK cells are capable of PAD4 citrullination enzyme expression, showing their possible role in the citrullination processes leading to production of RA-specific autoantigens.

GN4C was used for *in vitro* study of the expression of glycosyltransferases in NK cells. The glycomimetic was found to interfere with the glycosylation processes downmodulating the expression of both MGAT3 and MGAT5 which was proven in both fresh NK cells of healthy donors and NK-92 cell line. In both models we also observed an increase of cytotoxicity and the stimulation of Th1 cytokines – TNF-α, IFN-γ and particularly IL-2 promoting NK cell cytotoxic activity.

In the mouse model of RA we confirmed the gradual impairment of NK cell activity similar to human RA, corresponding with NK cell relative distribution. GN4C proved to be more potent in terms of amelioration of the clinical symptoms, reduction of inflammatory synovial infiltration as well as suppression of activated APCs, necessary for autoreactive T cell activation. Evaluation of the humoral immune response to the treatment showed inhibition of IFN-γ and TNF-α rise and stimulation of IL-4 corresponding with a reduction of CII-specific IgG2a.

This work brings a comprehensive overview of NK cell involvement in RA and the role of glycans in different *in vitro* and *in vivo* conditions. NK cells are able to react to specific RA autoantigens and participate in the citrullination processes leading to the progression of RA. The use of multivalent glycodendrimers *in vivo* is, however, capable of inhibition of the autoimmune processes bringing significant alleviation of CIA clinical symptoms in mice, suggesting potential new prospects for more effective therapeutic interventions in the early stages of RA.

1. Introduction

1.1 Autoimmune disorders

The mission of the immune system is not only to discriminate self from non-self but also to regulate and sustain weak self-reactivity as a necessary condition for immune homeostasis [1]. When disrupted, a pathological reaction against "self" may appear, causing a number of various conditions referred to as autoimmune diseases. Autoimmunity can be viewed as a defect of dendritic cells or aberrant B and/or T lymphocyte selection leading to a breach of autotolerance with development of immune reactivity towards native antigens [2, 3].

Such disorders can be generally divided into two basic types – local and systemic. While local autoimmunities (diabetes mellitus type 1, autoimmune thyroiditis, coeliac disease, myasthenia gravis etc.) affect only a specific organ or tissue [4-7], systemic autoimmune disorders including Rheumatoid arthritis (RA), Systemic lupus erythematosus or Dermatomyositis (DM) are associated with non-tissue-specific antigens [8-10]. In recent years, it has been shown that although a number of diseases are considered autoimmune, the specific immunopathology behind remains elusive. That is why the respective disorders should be viewed with regard to "immunological disease continuum" covering both the classical autoimmunities and autoinflammatory diseases driven by aberrant innate immune response as well as diseases that stand in between the two extremes [3].

Autoimmunity is a worldwide problem. In the US, the incidence varies depending on the individual disease ranging from 1 (systemic sclerosis) to more than 20 (rheumatoid arthritis) newly diagnosed patients per 100,000 person-years. Prevalence of the more common autoimmune disorders may reach up to more than 500 per 100,000 people [11].

1.2 Rheumatoid arthritis

Rheumatoid arthritis is one of the most prevalent systemic autoimmune diseases in the world. In Europe, its prevalence ranges from 310 to 860 cases per 100,000 [11-16] with 75% of the patients being women [11]. RA affects particularly synovia causing a chronic inflammatory condition resulting in a loss of function of the joints. When untreated at an early stage, the joint swelling stretches the surrounding tendons and ligaments, leading to characteristic articular deformities often seen in older patients. Lifespan of the patients is also shortened by as much as 12 years [17].

1.2.1 Pathogenesis of RA

Although the cause of RA still remains unclear, a number of studies described various parts of its pathogenesis. RA-associated antigens are presented to T cells by professional Antigen-presenting cells (APCs) carrying HLA-DRB1*04 cluster in 80%

of the cases [18]. In a healthy individual, T-cell overreaction is limited by a number of suppressing pathways such as CTLA-4 co-inhibitory molecule or regulatory T-cell (Treg) action. In RA, Treg function is negatively affected which helps the development of the disease [19]. The majority of T cells in the synovium usually comes from the CD4-positive (Th) subset. Their role in the pathogenesis of RA is the secretion of pro-inflammatory cytokines such as IL-2 and IFN-γ leading to the activation of macrophages and B cells as well as fibroblasts [20, 21]. Activated B cells then differentiate into plasma cells that secrete antibodies including autoantibodies.

Patients with RA are known to be positive for two basic types of autoantibodies. The classic diagnostic targets are so called rheumatoid factors (RFs) – IgM and IgA isotype antibodies directed against the Fc fragment of IgG. As these factors are not present in a significant number of RA patients and also do not directly contribute to the disease progression by means of cartilage damage, it seems that the other type of RA-related autoantibodies – ACPA (Anti-Citrullinated Peptide Antibodies) may serve as a better diagnostic and prognostic marker. There are 4 different ACPAs used in clinical practice – anti-CCP1-3 (cyclic citrullinated peptide) and anti-MCV (mutated citrullinated vimentin) with similar diagnostic and prognostic qualities. Table 1 shows the positive and negative predictive values of all the autoantibodies, their sensitivity and specificity. It is clear that although the presence of autoantibodies is important for the development of the disease, they cannot serve as the one and only diagnostic marker due to their limited sensitivity [22].

Autoantibody test	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)
Anti-CCP-2	67.1	79.0	50.0	88.4
Anti-CCP-3	64.0	80.0	56.4	84.6
Anti-MCV	56.3	79.2	57.4	78.4
RF	61.7	77.8	47.7	86.1

Table 1: *Comparison of rheumatoid autoantibody tests for predicting disease progression from undifferentiated arthritis (UA) to rheumatoid arthritis (RA). Adapted from Van der Linden et al., Arth Rheum 2009 [22]. Positive predictive value (PPV = true pos./all pos.); Negative predictive value (NPV = true neg./all neg.); Sensitivity (true pos./all RA); Specificity (true neg./all non-RA).*

The autoantibody response in RA varies over time being rather limited at the early stages of the disease, while a number of epitopes appears during the disease progression leading to production of a variety of antibodies with different specificities and isotypes [23, 24]. ACPA production during RA is used for discrimination

between two subtypes of the disease – ACPA-positive and -negative. ACPA-positive patients have larger lymphocytic synovial infiltrate and show increased joint damage and lower remission rates [25, 26].

Autoantibodies form immune complexes that reinforce the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF) via complement and Fc-receptor activation [27]. TNF is one of the key inflammatory modulators of RA. Its overproduction drives inflammatory processes leading to joint destruction as well as stimulates other pro-inflammatory cytokines such as IL-6 [28, 29]. The main source of proinflammatory cytokines are probably the macrophage-like synoviocytes. The ongoing inflammation leads to the destruction of cartilage as well as bone erosion caused by osteoclast activation, revealing epitopes for specific autoimmune response (for overview of the pathological processes and cytokine involvement see Fig. 1) [30].

1.2.2 Treatment of RA

RA can be treated either on the symptomatic level or by disease-modifying antirheumatic drugs (DMARDs). Symptomatic treatment usually utilizes non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics to reduce pain and stiffness. As NSAIDs show gastrointestinal and cardiac toxicity and their effect is rather limited, they are gradually losing their historical role as the first-line treatment [31, 32]. DMARDs are, on the other hand, becoming the mainstay treatment for RA [33]. They are a diverse group of compounds affecting various parts of the RA pathology and leading to reduction of the symptoms as well as function improvement and limitation of progressive joint damage [30]. The most widely used DMARDs are methotrexate, sulfasalazine or leflunomide [34-36]. DMARDs are often used in a combination therapy, however as they also have adverse effects (usually greater when used in combination) ranging from nausea to e.g. hepatotoxicity, continuous monitoring of the patients is required [37, 38].

Glucocorticoids are another treatment option. They, however, incur substantial adverse effects ranging from susceptibility to infections to osteoporosis, so a long-term use of these agents is generally considered unfavourable [39]. On the other hand, they can be beneficial in short-term use to moderate acute outburst of RA leading to rapid improvement. As DMARDs have slower onset of the effect, the use of glucocorticoids is an option of choice when it is necessary to reduce the symptoms quickly before DMARDs can be used.

A modern option for RA-suffering patients is the biological treatment. Anti-TNF antibodies were the first to be used in the clinical practice with significant results presented in a number of studies [40-43]. The most widely used Anti-TNF agents are Adalimumab, Infliximab or Etanercept (Anti-TNF-αR). Biologicals may be combined with conventional DMARDs (methotrexate, leflunomide) to increase efficacy [44]. The blockage of TNF by the biological treatment of course also brings concerns about side effects. The most prominent is the risk of infections ranging from bacterial

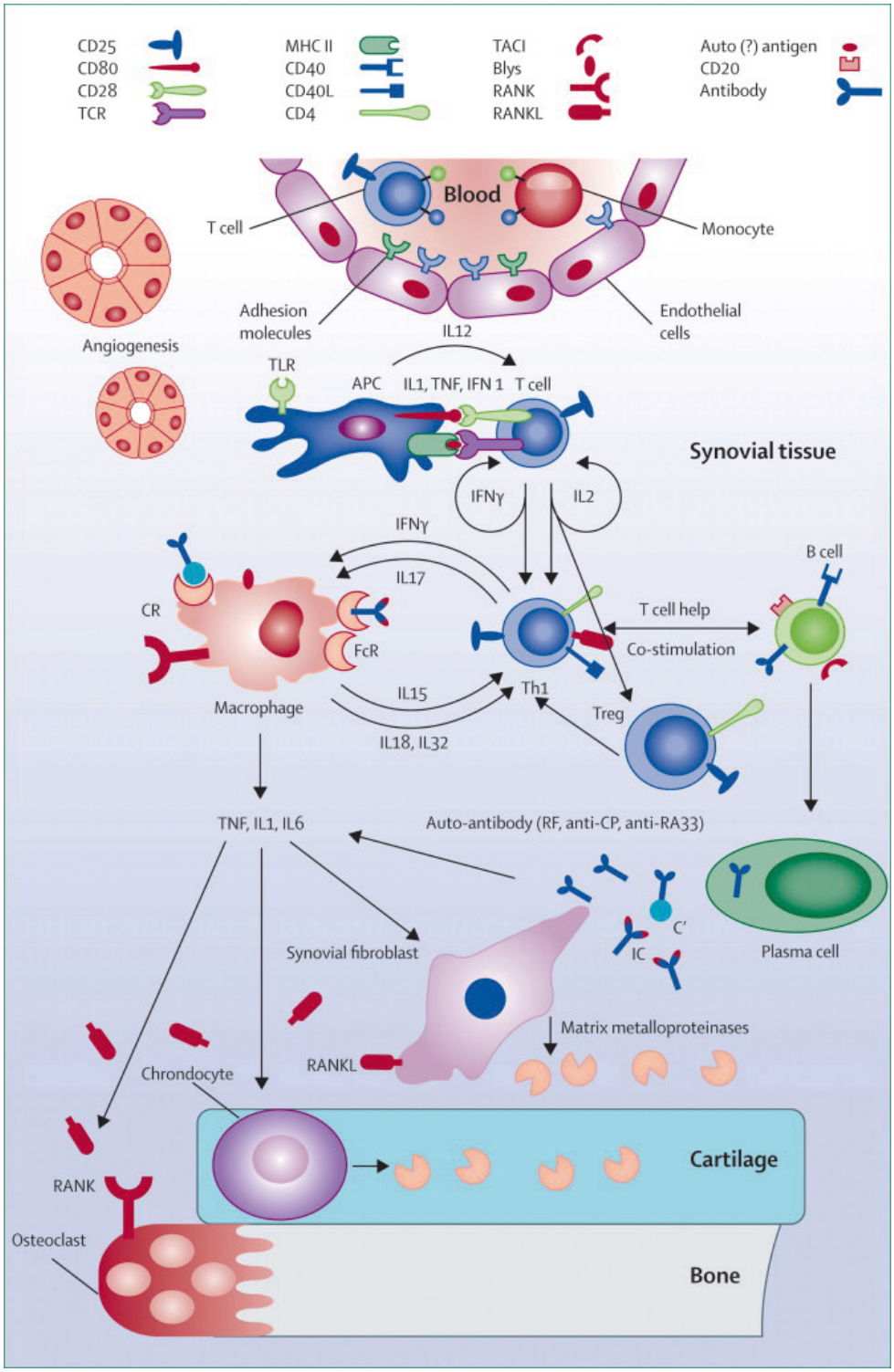

Figure 1: *Current views on the pathogenesis of rheumatoid arthritis (adopted from Smolen et al. Lancet 2007 370:1861-74 [21]). Arrows show some of many interactions in rheumatoid arthritis pathogenesis. Schematic depiction of events presumably occurring in synovial membrane, as well as articular cartilage and subchondral bone, which are surrounded by aggressive rheumatoid synovitis.*

(sepsis, abscesses) or fungal (candidiasis) to viral (herpes zoster) [30, 45]. New biological treatment modalities are thus being developed. They are targeted to the proximal effects of the immune response such as growth factors for T-cell subsets or inhibitors of Jak or Syk kinases [30, 46, 47].

1.3 Animal models of rheumatoid arthritis

Animal models of rheumatoid arthritis are widely used in both basic research and preclinical testing of potential anti-arthritic treatment modalities. Multiple models have been developed and the choice of which model to use depends on several criteria. In case of treatment testing, it is crucial that the results obtained from the model can predict the treatment efficacy in humans. In case of primary research of the immunopathology, it is important that the model shows similar pathological mechanisms leading to the establishment of the disease. In both cases, reproducibility as well as duration of the tests are important factors when choosing a suitable model [48]. As rodents are the most widely used laboratory animals, also arthritic models were developed mainly on rats and mice.

1.3.1 Rat RA models

One of the common models is rat adjuvant arthritis. It develops after injection of complete Freund's adjuvant (CFA) in the tail or paw of the animal. The disease onset appears approx. on day 9 after CFA injection in case of paw injection and both the acute inflammatory reaction and immunity-mediated inflammation of the contralateral paw (appearing later) can be observed [49]. Another rat model is the Rat Type II Collagen arthritis that appears after immunisation by homologous or heterologous type II collagen. The resulting symptoms include severe cartilage destruction with immune complex deposition [48, 50]. Although the pathology and resulting lesions of Type II Collagen rat arthritis resemble the situation in human RA more closely, pharmaceutical testing is more often performed on rat Adjuvant arthritis [51, 52].

1.3.2 Murine RA models

In mice, there are two commonly used model setups – collagen-induced arthritis (CIA) and collagen antibody-induced arthritis (CAIA). In the latter, the disease is elicited by injection of a cocktail of anti-collagen II monoclonal antibodies, followed by endotoxin. The advantages of this model are the rapid onset of the symptoms (the protocol takes 1–2 weeks), synchronicity and the possibility to use genetically-modified mice (transgenes and knockouts) [53]. CAIA is, however, a protocol only suitable for testing of treatment modalities affecting the symptoms of RA. As the symptoms are caused by direct injection of antibodies, this model lacks the whole ethiopathogenetic evolution preceding the appearance of the symptoms. When the target of the study is to describe the events leading to the disease or to test a treatment influencing development of the autoimmune reaction, CIA is a much more suitable model.

1.3.3 Collagen-induced arthritis

CIA provides the opportunity to study the nature of the autoimmune reactions leading to arthritis. Same as RA, CIA shows a chronic and progressive disease course and the susceptibility to CIA is correlated with certain MHC class II genes and the development of the disease is accompanied by T and B-cell response to type II collagen [54, 55]. CIA is elicited by injection of type II collagen emulsion with complete Freund's adjuvant (CFA). The mixture is injected intradermally into the proximal part of the tail of the genetically susceptible mice. The symptoms that appear in approx. three weeks include synovial hyperplasia, mononuclear cell infiltration or cartilage degradation, same as in RA. It is important to note that unlike in RA, there is no significant sex bias in CIA [56]. The disease is also usually monophasic, however, there were some relapsing mouse models described as well [57]. The original and well established strain used for the induction of CIA is the H2q bearing DBA/1 mouse. It has, however, been proven that also HLA-DR genes associated with the susceptibility to RA in human can be involved in the response to CII in transgenes [58, 59].

The immunopathology of CIA involves both T and B-cell response. The dominant determinants of CII for the interaction with T and B receptors have been described previously [60-64]. Unlike T-cell-mediated autoimmunities, such as experimental autoimmune encephalomyelitis (EAE), CIA is largely mediated by the anti-CII antibodies causing the complement-mediated cartilage destruction [56]. The similarities between CIA and RA make it an optimal animal model for both the basic research of the immunopathological processes and the drug-testing purposes.

1.4 Immune cells and their involvement in RA

1.4.1 Antigen-presenting cells

Pathology and ethiology of RA involves abnormal presentation of self-antigens [65]. The evidence lies in the association of the disease with certain HLA-DR chains [66, 67] and the rich presence of professional antigen-presenting cells (APC) in rheumatoid synovial membrane [68]. It was recently proved that HLA requirements of RA are associated with the preference of certain alleles to bind citrullinated peptides – – known RA autoantigens [69, 70]. Antigen presentation to T cells requires the interaction of CD28-CD80/CD86 costimulatory molecules [71]. These co receptors are expressed by various cells in the synovium at different levels. CD80 is being expressed only in low amount, while CD86 shows increased expression on a number of APCs including dendritic cells (DC), B cells and macrophages [72]. The expression profile of the costimulatory molecules with predominance of CD86 and production of IL-12 correlates with the preferred Th1 stimulation [73, 74].

Dendritic cells are among the most significant APCs. Under the influence of chemoattractants, they migrate to the synovial tissue where they receive differentiation signals from monocyte-derived cytokines and through CD40L interaction

with T cells [75, 76]. DCs are further stimulated by cytokines such as TNF-α and IL-1 present in the inflammatory conditions of the synovium. The major differentiation signal is, however, achieved through ligation of CD40, triggering the secretion of IL-12 and upregulation of CD80/CD86 and ICAM-1 co receptors [77]. Interaction of CD40-CD40L is also essential for the antigen-presenting function of B cells, particularly those producing RFs [78]. As these B cells carry an Fc-specific receptor, they serve as APCs for multiple antigens bound to different antibodies as well as induce T-cell help for RF secretion (see Fig. 2 for overview of antigen presentation in RA).

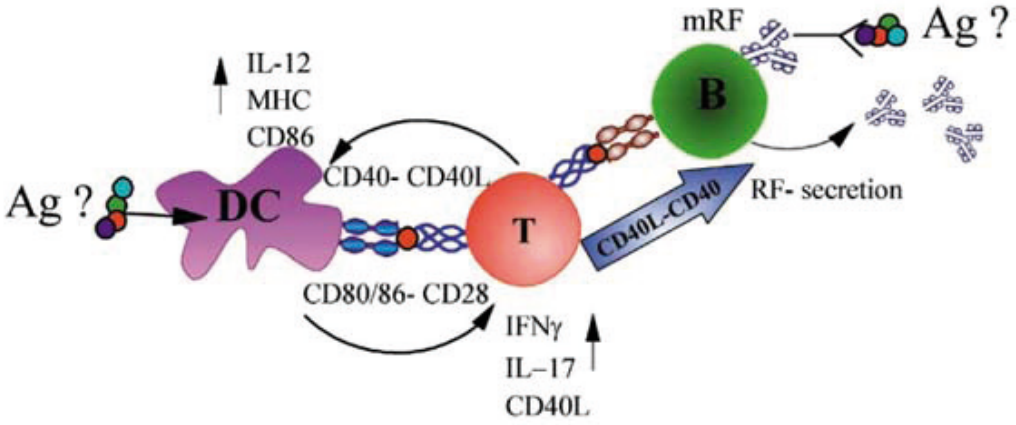

Figure 2: *APC-T cell interaction in the RA synovium (adopted from Aarvak and Natvig, Arthritis Res 2001 3:13-17 [74]). Dendritic cells (DCs) in the synovium present an external or local antigen to T cells. T cell receptor interaction and signalling via CD28-CD80/86 are essential for initial T cell activation leading to upregulation of CD40L on the T cells. Activated T cells are then capable of inducing further differentiation of DCs, as well as T cell help to rheumatoid factor B cells that have taken up antigen-Ig complexes. CD40-CD40L interaction between DCs, B cells and T cells may play a critical role in repeated activation of memory T cells in the synovium and, thus, maintenance of the inflammatory reactions.*

Synovial macrophages in RA are located particularly in the synovial lining where they are believed to have only lower importance as antigen-presenting cells as they are distant from the T cell-rich areas. They, however, show activated phenotype with increased expression of HLA-DR, CD86 and also STAT4 which indicates their role in IL-12 secretion and Th1 polarisation [79-81]. They were also found to be more efficient APCs than their counterparts isolated from peripheral blood [82].

1.4.2 B cells

B cells develop in the bone marrow where they progress through several stages of differentiation becoming immature B cells. These cells express surface IgM and migrate to peripheral lymph nodes [83]. There they are stimulated by antigens

to proliferate and express other antibody isotypes forming germinal centres and finally becoming plasma cells, secreting immunoglobulins. Formation of germinal centres is frequent in both generalized and organ-specific autoimmunities, being driven by inflammatory cytokines such as TNF-α [84, 85]. RA is hence no exception, showing abundant B cells within the inflammatory infiltrate that in approx. 30% of the patients form follicular lymphoid structures [84].

B cells play several critical roles in the pathogenesis of RA. These include production of RA-relevant antibodies mentioned earlier, secretion of cytokines and chemokines promoting inflammatory infiltration in the joints and also efficient antigen presentation and support of T cells [86]. This is particularly important, because owing to the high-affinity and specificity of their antigen-binding receptor, a specific B cell can present peptides with 1000-fold greater efficiency than other professional APCs. It has been already shown that T cell activation in RA depends on the function of B cells that cannot be effectively substituted by other APCs [87].

1.4.3 T cells

T cells are other key players in the (auto)immune response. They are a major subset of lymphocytes developing in the thymus and distinguished by the presence of antigen-specific T-cell receptor (TCR), CD3 complex and additional surface markers specific for individual subpopulations (CD4 for helper T cells (Th) and CD8 for cytotoxic T cells (Tc, CTL)) [88].

The function of Th cells is closely influenced by antigen presentation by either B cells or other APCs. Th cells require three different stimuli from the APC to become fully functioning and perform their task (see Fig. 3). The first stimulus is the presentation of the antigen on MHC class II molecule binding the specific TCR. To expand the Th cell population, a costimulatory signal is necessary in the form of CD80/CD86 binding CD28 co-receptor on the surface of the Th cell. Finally, the third signal comes in the form of cytokine secretion by the APC. This last signal is absolutely crucial for the determination of the type of immune response, leading to either cell-mediated (Th1) or humoral (Th2) response [89].

Under autoimmune conditions, the destiny of the autoreactive Th cells also relies on the cytokines secreted by the APCs. These cytokines drive Th cells into either Th1 or Th2 phenotype, which have distinct effector functions. While Th1 cells secrete predominantly IL-2, IL-3, TNF-α and particularly IFN-γ and push the immune response towards cell-mediated function such as the activation of macrophages and CTLs, Th2 cells secrete rather different spectrum of cytokines including IL-4, IL-5 and IL-13, stimulating B cell activation, antibody production and class switching [90]. Interestingly, the cytokines of a particular Th subtype are able to further promote the expansion of that population while simultaneously inhibiting

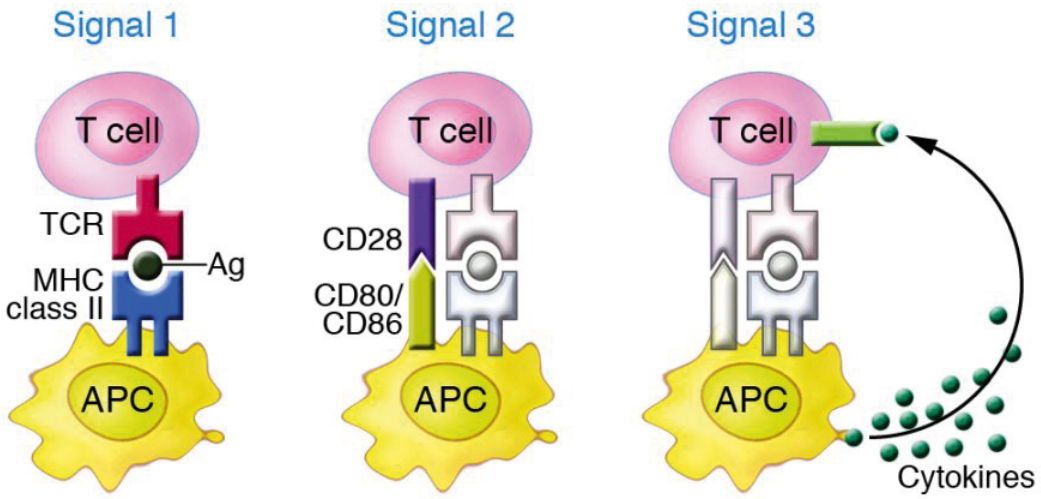

Figure 3: *Three signals necessary for successful T cell activation (adopted from Gutcher and Becher, J Clin Invest 2007 5:1119-1127 [89]). Signal 1 comprises the presentation of antigen peptide in the context of MHC class II molecules which is recognized by the antigen-specific TCR. Signal 2 involves the stabilization of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells. CD80/CD86 on APCs interact with their receptor, CD28, on T cells to generate activating signals, while interaction with cytotoxic T lymphocyte–associated protein 4 (CTLA4) generates inhibitory signals (not shown). Signal 3 is produced by the secretion of cytokines by APCs, which signal via cytokine receptors on T cells in order to polarize them toward an effector phenotype.*

the development of the other subset. This allows each TH subset to produce characteristic cytokines that in turn provoke the development of a distinctive effector function specific for that immunogen [91].

A rather novel subtype of TH lymphocytes are the TH17 cells. These are major producers of IL 17 – an inflammatory cytokine that has recently been believed to mediate a number of pathological processes in autoimmunity including RA [92, 93]. IL-17 produced by TH17 cells induces the expression of IL-1, IL-6 and G-CSF as well as chemokines by fibroblasts, stromal or endothelial cells at the site of inflammation [94, 95].

Cytotoxic T lymphocytes (Tc, CTL) are the other major subtype of T cells. They are best known for their role in the destruction of transformed or infected host cells. They carry CD8 co receptor on their surface and recognize peptide fragments on MHC class I proteins. As their effector function is based on direct cell-cell interaction, their presence in situ is essential [96]. Although the pathological role of TC cells was proved in autoimmune diseases such as multiple sclerosis, encephalomyelitis or diabetes mellitus [97-99], its role in RA is rather unclear with evidence that they can both promote and protect from synovial inflammation (reviewed by Carvalheiro et al. [100]).

Recently, it has become clear that RA pathogenesis cannot be explained solely by the classical antigen-driven expansion of effector T cells targeting the synovia, although Th1 cells expressing IFN-γ and TNF-α are detected in the synovia during established disease, simultaneously lacking the expression of IL-2 and IL-4 [101-103]. There is a striking characteristic of the rheumatoid synovium carrying inflammatory infiltrates that resemble tertiary lymphoid structures showing germinal centre-like reactions [104]. This fact provides evidence of ongoing immune reaction in situ with the possibility that T cells influence not only B cells and subsequent autoantibody production but also local macrophages and stromal fibroblasts, promoting cytokine and chemokine expression via direct cell-cell interaction [105].

1.4.4 NK cells

The attention of the autoimmune community is currently being focused on the involvement of natural killer (NK) cells in RA as well as other autoimmune diseases. They are present in a number of locations such as blood, bone marrow, spleen, liver, lymph nodes, thymus, lung, peritoneum or in the uterus during gestation [106]. They were originally defined by their ability to spontaneously kill tumour cells and virus-infected targets without the necessity of prior activation [107, 108]. Currently, they are known to be capable of recognizing and destroying a number of other targets such as transplanted, antibody-coated or stressed cells [109]. Although their primary function is cytotoxicity, NK cells also possess an important immunoregulatory function through cytokine secretion [110], priming the adaptive immune response of T and B cells or activating dendritic cells (DCs) [111-113].

In humans, NK cells are defined by the expression of CD56 and simultaneous absence of CD3 (CD3–CD56+). They, however, further divide into two distinct subsets – cytotoxic and regulatory NK cells. Cytotoxic NK cells, representing 90% of all NK cells and prevalent in the peripheral blood, are defined as CD56dimCD16bright. Regulatory, cytokine-producing, NK cells show CD56brightCD16– phenotype and represent approx. 1% of peripheral blood cells [114]. In mice the situation is more complicated. Murine NK cells can be defined by the presence of either CD49b and absence of CD3 (CD3–CD49b+) or the sole presence of NKp46 marker which is, unlike CD49b, not present on NKT cells [115, 116]. As murine NK cells do not express CD56, it is not possible to easily distinguish between cytotoxic and regulatory NK subsets. Hayakawa et al., however, suggested the expression of CD27 marker to define cytokine-producing murine NK cells [117, 118].

NK cells themselves are activated by the presence of cytokines such as IL-2 or IL-15. They are capable of antibody-dependent cell-mediated cytotoxicity (ADCC) utilizing CD16 (Fcγ receptor IIIA) on their surface binding the Fc antibody part. Cytotoxicity itself is mediated by the release of cytoplasmic granules containing perforin and granzyme. Perforin forms a pore in the cytoplasmic membrane of the target cell, letting granzyme enter and induce apoptosis. Constitutive production of perforin within NK cells is largely enhanced by IL-2 stimulation [119, 120]. Other pathways

of NK-mediated cell lysis are TRAIL (TNF-related apoptosis-inducing ligand) or Fas ligand expression. Both systems are capable of apoptosis induction in the target cells and their expression is stimulated by IL-2 or IL-15 [120].

The second important function of particularly cytokine-producing NK cells is the immune regulation performed particularly via cytokine production, which is critically important for both innate and adaptive immune responses. The major cytokines of NK cells are IFN-γ and TNF-α [121, 122] that have pro-inflammatory function, induce Th1 response and MHC-I expression on a number of cells [106]. Subsets of NK cells have, however, been found to express also GM-CSF, IL-5, IL-10, IL-13, IL-22 or TGF-β [109, 120, 123, 124].

NK cell function and response is regulated by a varied repertoire of activating and inhibitory receptors. The interplay between them and the prevailing of either activating or inhibitory signals lead to the possible initiation of cytotoxic function [125, 126]. Unlike specific immune receptors (TCR or BCR), the genes that encode NK receptors do not undergo recombination [106]. A number of NK receptors monitor MHC-I expression. These belong under killer immunoglobulin-like family (KIR), Ly49 or CD94/NKG2 groups. Other, activating, receptors such as NKG2D recognize MHC-like molecules such as MICA, MICB in human or RAE1 ligands in mice [120].

NKG2D is an important activating receptor of virtually all NK but also CD8+ αβT, subset of γδT and most NKT cells. It has also been reported on human CD4+ T cells but primarily under pathological conditions including rheumatoid arthritis [127, 128]. In mice, NKG2D is, apart from other cell types, expressed on activated and memory CD8+ αβT cells. The receptor is encoded by Nkg2d (Klrk1) gene located in the NKC gene complex on chromosome 6 in mice and ch. 12 in humans [129, 130]. It has been shown to have considerable importance in a variety of innate immune responses related to cancer or autoimmunity [114, 131, 132]. NKG2D receptor mediates "induced-self recognition" which refers to markers upregulated on stressed cells [133]. Unlike other members of the NKG2 family, NKG2D does not form a heterodimer with CD94 and does not recognize MHC class I molecules (HLA-E in humans or Qa1 in mice) [134]. Human ligands include MHC-I-like molecules MICA and MICB and UL-16-binding proteins ULBP1-4. In mice, NKG2D recognizes RAET1 ligands (retinoic acid early transcript 1) such as RAE1α–ε, H60 or MULT1 [109, 120].

Inappropriate or deregulated expression of NKG2D was previously reported to break the delicate balance between immune activation and tolerance and trigger autoimmune responses [135]. These include coeliac disease, Crohn's disease, type 1 diabetes but also RA. High levels of IL-15 and TNF-α in the sera and joints of RA patients induce aberrant expression of NKG2D on CD4+CD28– T cells. These are subsequently activated in an NKG2D-dependent manner as MICA and MICB ligands are also vastly upregulated in the synoviocytes [127].

NKR-P1 family receptors present on a subset of NK and T cells were first characterized in the rat but later also murine and human analogues were discovered. Murine NKR-P1 family comprises -A, -B, -C, -D, -F and -G isoforms, while in humans, only one isoform – NKR-P1A (CD161) is present [136, 137]. It was found that NK1.1 gene is, along with other NK antigens (such as CD69, CD94-NKG2D complex, human KIRs or rodent Ly49 [138]), mapped to a single region of distal chromosome 6, later denominated as the NK gene complex or NKC [139, 140]. The NKR-P1 receptors are approx. 60kDa homodimeric disulphide-linked C-type lectin-like molecules [136, 141]. NKR-P1C is an important activating receptor of NK cells in mice. Its stimulation by antibody-mediated (clone PK136) crosslinking on C57Bl/6 NK cells lead to antibody-induced redirected lysis (AIRL) and IFN-γ production [122, 142]. It was later found that NKR-P1C is associated with FcRγ adaptor molecule also functioning in FcγRIII (CD16) or FcεRI signal transduction [143]. This adaptor molecule contains immunoreceptor tyrosine-based activation motif (ITAM) and the crosslinking of the receptor leads to recruitment of Syk kinase and subsequent activation of NK lytic activity [144, 145]. On the other hand NKR-P1B, another member of the NKR-P1 family, is an inhibitory receptor. Its inhibitory function exceeds the activating capabilities of NKR-P1C [144]. NKR-P1B contains immunoreceptor tyrosine-based inhibition motif (ITIM; LxYxxL) in its cytoplasmic domain that binds SHP-1 protein tyrosine phosphatase mediating the inhibitory function of the receptor [144, 146, 147]. Not many natural ligands of NKR-P1 family have been identified yet, leaving ligands of some of the activating isoforms (NKR-P1A, NKR-P1C) still unknown. The inhibitory NKR-P1B/D isoforms were found to bind Ocil/Clr-b (osteoclast inhibitory lectin) while stimulatory NKR-P1F was reported to bind Ocilrp2/Clr-g/Dcl-1/LCL-1 [148, 149]. In humans, the only member of the NKR-P1 family is the -A isoform being also referred to as KLRB1 or CD161. It is an inhibitory receptor sharing 45% sequence homology with its murine analogues. Its known functional ligand is Lectin-like transcript-1 (LLT1) [150, 151].

The role of NK cells in RA is unclear with reports that it may either contribute to, or protect against inflammation. Studies regarding peripheral blood NK cells are not conclusive as contradictory data concerning the changes of NK cell percentage were published. Most of the authors, however, reported impairment of NK cytotoxicity in the course of RA [152, 153]. The synovium of RA patients contains a number of inflammatory cells ranging from innate immune cells such as macrophages, neutrophils or dendritic cells to antigen-specific T, B and plasma cells [154]. It was shown that NK cells are also present in the synovial fluid of RA patients and that they exhibit a CD56bright CD94bright CD158– phenotype which suggests their cytokine-producing nature [155, 156]. In early RA, NK cells present in the synovium also show an increased expression of granzyme compared to the situation in osteoarthritis [157]. This is an important finding as granzyme may play a role in the development of autoimmunity producing new epitopes and promoting cartilage destruction [158].

NK cells, and particularly CD56bright subset, are a major source of IFN-γ, a generally considered pro-inflammatory cytokine that was, however, reported to have also some anti-inflammatory effects [154]. A major role of IFN-γ is the antibody class switch towards IgG2a isotype and it also plays important roles in DC maturation. However, it also suppresses Th17 differentiation and was reported to attenuate autoinflammatory conditions in RA or CIA [159, 160]. Cytokine-producing NK cell subset is also influenced by a number of Th1 cytokines such as IL-12, IL-15 or IL-18 that are present in the inflamed joint environment. These can induce production of pro-inflammatory cytokines such as TNF in the CD56bright NK cells [161]. The effects of the intricate cytokine milieu within the synovial tissue are also closely connected to the cell-cell interaction between NK cells and other immune cell types. NK cells present in the synovial membrane can provide costimulatory signals to T and B cells [159]. Dalbeth et al. showed that NK cells reciprocally communicate with monocytes on the cell-contact basis and stimulate the production of TNF in monocytes and, on the other hand, get activated [162]. Moreover, both peripheral blood and synovial NK cells stimulate differentiation of monocytes into dendritic cells, leading to induction of T cell activation and Th1 polarisation [163]. Conversely, NK cells are also capable of killing immature DCs, only showing the complexity of all the possible interactions [164]. NK cells were also reported to interact with the fibroblast-like synoviocytes, leading to secretion of IL-15 by the synoviocytes and proliferation and stimulation of NK cells. Such effects may lead to promotion and/or maintenance of inflammation [165].

1.5 Glycosylation and autoimmunity

Protein glycosylation is a crucial process in the living organism. Almost all secreted and surface proteins undergo glycosylation and contain significant mass of covalently attached glycans [166]. The amount of carbohydrates can represent more than 50% of the glycoprotein molecular mass [167]. The carbohydrates attached to the protein fall into two categories – N linked and O-linked depending on the nature of the linkage [166]. The repertoire of possible glycan structures is estimated to contain thousands of variants generated by a group of glycosyltransferases and glycosidases [168]. Such complexity of glycosylation often leads to ignoring it as a "complicating factor" in the research of protein structure and function. This heterogeneity may, however, be the key to understanding a number of processes in both health and disease.

In the immune system, glycosylation plays a crucial role. Almost all of the surface receptors on immunocytes are glycosylated. These include pattern-recognition receptors, toll-like receptors, class I and II MHC proteins, receptors for cytokines and chemokines as well as T and B cell receptors and their co-receptors [166]. The role of glycosylation here is as varied as the functions of the receptors themselves and its influencing leads to significant changes in the immune response and regulation. For example, prevention of proper TCR glycosylation substantially eases T cell activation [169] or improper N-glycosylation of MHC II significantly impedes the ability to present bacterial polysaccharide antigens [170]. Secreted glycoproteins include

essentially all cytokines, chemokines, a number of complement components and also antibodies. Glycosylation of Fc portion of antibodies may even affect their function sending either activating or inhibitory signals to the responding immune cells [171].

Concerning the mentioned extent of glycosylation effects in the immune system, it is no surprise that autoimmune reactions are also vastly affected, though the awareness of the research community of such effects is relatively new. These data are based on observations describing remodelling of surface glycans in the transition from normal to inflamed tissues [172]. These often influence trafficking of immunocytes to the sites of inflammation representing a promising target for anti-inflammatory therapy [173].

Glycosylation can, however, control autoimmune response also in other ways. In rheumatoid arthritis, effector T cells express altered cell surface glycans, especially those exposing terminal GalNAc and Gal-β(1-4)-GlcNAc structures [174]. Glycosylation was also proved to affect the threshold of TCR activation having profound effects on T-cell function during both healthy and pathological cellular responses. MGAT5 (GnT5) glycosyltransferase initiates formation of β1–6-N-glycan-branch structure on various glycoproteins including the TCR. T cells lacking this enzyme have a decreased activation threshold and GNT5-deficient mice have increased susceptibility to autoimmunity such as EAE [169]. Grigorian et al. did, however, show that treatment with high doses of GlcNAc causes an increase of GnT5-mediated N-glycan branching and inhibits the TCR overactivation [175].

Although a number of studies showed the effect of glycosylation on adaptive immune responses, there is also evidence of the role of glycosylation in innate immune contribution to the development of autoimmunity and inflammation. In mice, the deficiency in α mannosidase-II (αM-II), responsible for complex-type N-glycan branching, leads to an autoimmune condition resembling human systemic lupus erythematosus. This effect was solely linked to chronic activation of innate immune components, independently on the adaptive immune response. The proposed mechanism considered exposure of cryptic N-glycans under αM-II deficiency resulting in recognition by endogenous lectins leading to aberrant activation of innate immune responses [176].

2. Aims

The primary objective of this work was to provide new insights into the pathology of autoimmunity, and particularly rheumatoid arthritis, with special regard to innate immune responses and its particular role in the progression of the disease. Such information is necessary for development of novel therapeutic approaches and strategies bringing beneficial effects for the patient. In order to contribute to the current knowledge in the field of rheumatic immunopathology, we focused on the following:

- **Analysis of NK cell function in human rheumatoid arthritis and its modulation by rheumatic autoantigens as well as glycans**
- **Description of the effect of GN4C multivalent glycodendrimer on NK cells and their function *in vitro***
- **The *in vivo* effect of GlcNAc in the form of multivalent dendrimers as a treatment modality in an animal model of rheumatoid arthritis**

3. Publications

3.1 CD161 receptor participates in both impairing NK cell cytotoxicity and the response to glycans and vimentin in patients with rheumatoid arthritis

3.1.1 Overview

Rheumatoid arthritis is a chronic inflammatory disease affecting synovial tissue in multiple joints. Inflammation is mediated by autoreactive T cells, activated macrophages and synoviocytes, stimulating production of inflammatory cytokines. Innate immunity is of critical importance here as well, however the exact mechanisms of its involvement are often unknown or poorly described. NK cells assist in the initiation and development of adaptive immune responses by their cytokine production or direct cell-cell interaction. Their impaired function may thus disrupt the fine balance between immune tolerance and response and cause persistence of autoreactive T cells. One of the major NK cell receptors is CD161. Its activation sends inhibitory signals that attenuate NK cell response. CD161 bind LLT1, or osteoclast inhibitory lectin (CLEC2D), that is known to inhibit osteoclastogenesis. It is also present on activated B cells mediating direct interactions with NK cells [150, 177, 178].

RA is at least in part a deregulated-glycosylation disease as changes appear in the glycosylation profile of cartilage and synoviocytes as well as other structures [179, 180]. Dense and complex glycan structures are the most abundant and diverse molecules on cell surfaces and their patterns are modified by Golgi-resident glycosyltransferases such as MGAT5, required for the generation of N-glycan complexes. Alteration of their function resulting in impaired N-glycan branching, together with antibodies recognizing endogenous glycans are associated with multiple autoimmune disorders [181, 182].

Currently, the only specific marker for RA is the presence of citrullinated proteins that function as autoantigens [183, 184]. PAD2 and PAD4 citrullination enzymes are, under normal conditions, intracellular, however, in the inflamed synovium, they may leak and become active in the extracellular space producing extracellular citrullinated epitopes.

3.1.2 Aims and methods

In this study, we dealt with functional impairment of NK cells reported in RA as well as other systemic autoimmune disorders. We performed an extensive study on 146 patients and healthy donors (RA n=50; OA n=19; DM n=12; PM n=5; healthy donors n=60). We used direct cytometric and cytotoxicity analysis of blood and synovial fluid samples as well as *in vitro* cultivation, molecular techniques or ELISA to describe the status of NK cells in the inflammatory environment as well as the reaction to dendrimeric GlcNAc complexes or the disease-specific autoantigen (vimentin).

3.1.3 Results and discussion

NK cells preserve immunological tolerance by providing negative feedback to a number of cell types incl. autoreactive T and B cells [185]. We examined NK cytotoxicity in 2 different autoimmune conditions – RA and PM/DM (polymyositis/dermatomyositis), OA (osteoarthritis) and HD (healthy donors). In both autoimmunities (RA and PM/DM), the NK function was impaired, however, in RA, it did not correlate with reduced numbers of NK cells. We tested the *in vitro* reaction to GN8P glycoconjugate as a prototype glycan structure and divided the samples into GN8P-inhibited and –nonreactive groups. Subsequently we found out that the GN8P-inhibited samples showed significantly higher expression of CD161 inhibitory receptor. Statistical analysis showed that 2/3 of the patients with active disease fall into the CD161 high-expressing group, suggesting the importance of CD161 molecule binding epitopes present under the inflammatory conditions and leading to NK inhibition. Concerning the therapeutic outcome with regard to our results (inhibition of NK cell function), we found combined therapy with glucocorticoids and immunosuppressive drugs more effective than other treatment types (anti TNF therapy or monotherapies).

To explore the mechanisms underlying glycosylation pathways, we examined the effect of GN8P on MGAT5 expression. GN8P can serve as a substrate for MGAT5, responsible for GlcNAc branching, playing a key role in autoimmunity development [186, 187]. We found increased level of MGAT5 in the synoviocytes of the patients but not in their peripheral blood mononuclear cells (PBMC). This result indicates that GlcNAc terminated glycans may be involved in the upregulation of CD161 expression and result in downmodulation of NK cell function.

A characteristic feature of RA is the reaction of lymphocytes to the MCV (mutated citrullinated vimentin) autoantigen. We tested the expression of CD161 by NK cells after such *in vitro* treatment. In healthy donors, we observed an increase of CD161 expression, while in RA patients, where MCV had been present in the environment, there was no further increase of CD161 observed *in vitro*. The increased CD161 expression in RA conditions may be due to the continual stimulation by MCV in situ. We also showed that NK cells are capable of reaction to MCV by upregulation of PAD4 expression. This was again observed only in healthy donors. These results suggest that NK cells may be involved in the citrullination processes participating in RA pathogenesis.

Taken together, we discovered that NK cells recognize RA autoantigen MCV that may serve as a triggering mechanism for CD161 upregulation. We proved that CD161 expression correlates with the impaired NK cell function in RA inflammatory conditions that, as we suggest, leads to inadequate attenuation of autoreactive T cells, promoting disease progression.

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CD161 receptor participates in both impairing NK cell cytotoxicity and the response to glycans and vimentin in patients with rheumatoid arthritis

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Abstract We investigated the role of natural killer (NK) cells and CD161, their primary C-type-lectin-like receptor in rheumatoid arthritis (RA). Samples were compared with healthy donors (HD), dermatomyositic (DM), polymyositic (PM), and osteoarthritic (OA) patients. RA, PM, and DM NK cell cytotoxicities significantly decreased relative to the HD and OA NK cells ($p<0.0001$). These results correlated with an increased expression of NK cell inhibitory receptor CD161, in active disease RA patients. We demonstrated that NK cells are able to respond to mutated citrullinated vimentin (MCV), an RA-specific autoantigen, leading to increases in both PAD4 enzyme and CD161 mRNA expression. MGAT5 glycosidase involvement was detected in GlcNAc metabolism within the synoviocytes of RA patients. Our findings reveal a functional relationship between CD161 expression and NK cell cytotoxicity as well as reactivity to glycans and MCV, thus providing new insight into the pathogenesis of RA and confirming the involvement of surface glycosylation.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects the synovial tissue in multiple joints. Inflammation in RA and other rheumatoid diseases is mediated by activation of T cells, leading to activation of macrophages and fibroblast-like synoviocytes that subsequently produce proinflammatory cytokines. Synovial tissue proliferation is associated with destruction of cartilage and bone; the closely related production of proteinases (MMPs), which degrade collagen and proteoglycans, serves to further antigenically challenge the immune system [1].

Innate immunity is likely to be of critical importance in the development and regulation of autoimmunity. However, the cells, receptors, and mediators involved in various autoimmune conditions are mostly unknown. Natural killer

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(NK) cells are the key players in innate immunity; in addition to their primary function of killing virally infected or transformed cells, they also assist in the initiation and development of adaptive immune responses by producing cytokines or by direct cell-to-cell interactions. Reduced numbers or impaired functioning of NK cells could lead to the persistence of autoreactive T cells. Therefore, it is important to identify NK cell activity and related molecular events in RA patients. NK cell function is tightly regulated by activating and inhibitory signals that are delivered by a diverse array of cell surface receptors [2]. One important inhibitory receptor on human NK cells is CD161 (NKR-P1A), which belongs to the C-type lectin family that preferentially binds carbohydrate-bearing structures. In a previous study, we determined that CD161 binds to an N-acetylglucosamine (GlcNAc)-containing glycoconjugate [3].One of the described natural glycoprotein ligands of CD161 is LLT1 (osteoclast inhibitory lectin or CLEC2D) that is known to inhibit in vitro osteoclastogenesis [4,5]. LLT1 is present on the surfaces of dendritic and activated B cells, and plays a role in mediating direct interactions between these cells and NK cells [6]. Although previous studies have investigated the role of some CD161 ligands, little is known about the function of the CD161 receptor and its role in autoimmune diseases.

RA is, at least in part, a deregulated glycosylation disease, and cells from patients with the disease display changes in the glycosylation of cartilage and synoviocytes. Changes appear also in the glycosylation or expression of galectins, lectins, acute-phase proteins, IgG, and other unidentified plasma proteins [7–10]. Dense, complex glycan structures are the most abundant and diverse molecules on cell surfaces. The glycoprotein pattern is modified by Golgi-resident glycosyltransferases such as MGAT5, which is required for the generation of N-glycan complexes. These enzymes are regulated and expressed in a tissue-specific manner that enables them to rapidly modify the glycan profile in response to physiological or pathological changes. Alterations in protein N-glycan branching, together with the presence of antibodies that recognize endogenous glycans, is associated with autoimmune disorders [11–13]. MGAT5-deficient mice have increased spontaneous kidney autoimmunity and increased susceptibility to experimental autoimmune encephalomyelitis [14]. Knowledge of Golgi-resident enzymes operating in glycan synthesis pathways could provide further insights into the pathogenesis of autoimmune diseases [15–17].

Currently, the only specific marker for RA is the presence of citrullinated proteins, which are produced in inflamed synovium and function as autoantigens (cit [18,19]). Anti-citrullinated protein antibodies circulating in the peripheral blood of RA patients can serve as highly specific markers for early detection of RA, and can be used to predict disease outcome [20–24]. Proteins are citrullinated by peptidylarginine deiminase enzymes (PADs). PAD2 and PAD4 are predominantly expressed in CD14+ macrophages and, to a lesser extent, in CD56+ cells [25]. Under normal conditions, PAD enzymes are located intracellularly; however, in inflamed synovium, they can leak from damaged cells and become active due to high extracellular Ca^{2+} levels [26].

Although it is known that NK cell-mediated cytotoxicity is downmodulated in systemic rheumatoid diseases [27], the molecular mechanism is not fully understood. Thus, we investigated the NK cell functional impairment that persists even after patients have received classical therapies to treat RA. We focused on the role of NK cells and CD161 in patients with RA, polymyositis (PM) and dermatomyositis (DM), and compared it with their roles in patients with osteoarthritis (OA), and in healthy donors (HD). Specifically, we evaluated saccharide recognition of the CD161 inhibitory receptor using synthetic N-acetyl-β-D-glucosamine-terminated glycoconjugates, as well as the possible interaction between CD161 and modified citrullinated vimentin (MCV).

Patients and methods

Patients and healthy donors

The study population comprised 86 patients (5 men and 81 women) followed by the Institute of Rheumatology in Prague, Czech Republic, and 60 healthy donors (HD, 24 men and 36 women, mean age 46.1 ± 9.2 years) from the Blood Transfusion Department of Thomayer University Hospital in Prague. Patients had the following diagnoses: RA (n = 50, mean age 59.3 ± 10.3 years), OA (n = 19, mean age 66.7 ± 6.3 years), DM (n = 12, mean age 61.4 ± 4.8 years), and PM (n = 5, mean age 60 ± 4.9 years). Patients with DM, or PM were diagnosed using valid criteria including MRI [28–30]. Patients with RA met the 1987 revised criteria of the American College of Rheumatology (ACR) [31]. Patients with OA were diagnosed according to the ACR Subcommittee on Osteoarthritis [32].

Peripheral blood or synovial fluid from patients and healthy donors was drawn after we obtained an informed consent agreement. Therapy involved the use of glucocorticoids, immunosuppressives (cyclosporin A, methotrexate, azathioprine, and leflunomide), TNF antagonists, and non-steroidal anti-inflammatory drugs. To exclude the influence of therapy on the immune parameters that we investigated, four untreated RA patients were also enrolled in the study.

Cell separation and glycoconjugate or vimentin treatment

Citrated blood samples from patients and healthy donors were separated by Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) density centrifugation for 40 min to obtain the peripheral blood mononuclear cell (PBMC) fraction. Synovial fluid including synoviocytes was obtained from patients with RA. Purified human CD56+CD3− NK cells (>98%) were sorted with a FACS Vantage SE (BD Bioscience, Heidelberg, Germany) using antibodies CD3-FITC (Dako, Glostrup, Denmark) and CD56-PE (Immunotech, Marseille, France).

A glycoconjugate–polyamidoamine core with eight terminal *N*-acetyl-β-D-glucosamine moieties (GN8P) was synthesized and kindly provided by Vladimir Kren (Academy of Sciences, Prague, Czech Republic). The synthesis and dose-dependent effects of this glycoconjugate on human PBMCs were described previously [33,34]. Purified NK cells were incubated with GN8P (10 nM), RA-specific autoantigen MCV (Orgentec, Mainz, Germany), and recombinant human vimentin (VIM, 10 mM; Progen Biotechnik, Heidelberg, Germany) at 37 °C for 24 h. GN8P and VIM were added to the cells in solution, and MCV was coated to microtitre

plates. Cell lysis was performed after incubation directly on the plates.

Cytotoxicity assay

In vitro NK cell-mediated cytotoxicity was determined using a standard ^{51}Cr-release assay with PBMCs from patients and healthy donors in the presence or absence of GN8P. We used the NK cell-sensitive K562 (CCL-243, ATCC) erythroleukemia cell line. Effector cells at a concentration of 1.6×10^5/well were incubated with 10^4 target cells (labeled for 60 min with $Na_2^{51}CrO_4$), in round-bottomed 96-well microtitre plates (NUNC) at 37 °C in a humidified atmosphere containing 5% CO_2. NK cell activity was evaluated after 18 h of incubation and was calculated as described previously [35].

Flow cytometry

The fluorochrome-conjugated antibodies CD3-Pacific Blue (clone UCHT1), CD56-APC (clone MEM-188), and CD161-FITC (clone B199.2) were obtained from Dako (Glostrup, Denmark), Exbio (Prague, Czech Republic), and Serotec (Raleigh, NC, USA), respectively. Optimal staining concentrations were determined by single-stain titration of antibodies prior to experimental use.

PBMCs (5×10^5 cells/well) were stained with the antibody mixture for 30 min on ice, washed, and measured with a Becton Dickinson (Franklin Lakes, NJ, USA) LSRII instrument. We included single-stain controls for further offline compensation. Measurement and subsequent analysis was performed using FACSDiva and TreeStar FlowJo 8 software, respectively.

Reverse transcriptase PCR and real-time reverse transcriptase PCR analyses

Total RNA from purified NK cells and PBMC was isolated with RNeasy Micro and Mini Kits (Qiagen, Hilden, Germany), respectively. Five micrograms of RNA were transcribed into cDNA using the cDNA Archive Kit (Applied Biosystem, Foster City, CA, USA). Reverse transcriptase PCR (RT-PCR) was carried out with HotStarTaq DNA Polymerase (Qiagen) and an iCycler5 (Bio-Rad, Philadelphia, PA, USA). Real-time RT-PCR was performed with PowerSybr Green Master Mix (Applied Biosystems) and an iCycler5. PCR product specificity was checked by melt curve analysis. The sequences of the primers used to amplify human β-2-microglobulin (B2M) and PAD4 were described previously [36,37]. The primers designed by us with Primer3 Input software were: MGAT5 F, 5′-CTTCTTTCTTCCAGCACCTCAAC; MGAT5 R, 5′-AAACACACAGTGCTTATTCTTAGGG; NKR-P1A F, 5′-TCTTCCTCGGGATGTCTGTC; and NKR-P1A R, 5′-CCTGCTCTGTTGAATGTCCA. The gene of interest was normalized to the control gene β-2-microglobulin and differences in gene expression between the treated cells and the appropriate control cells were evaluated with Bio-Rad iQ5 2.0 software.

ELISA to detect MCV and CCP antibodies

An anti-MCV ELISA kit (Orgentec, Mainz, Germany) was employed to detect antibodies against MCV. Antibody amounts were calculated as pg/ml on the basis of the standard curve. Antibodies to citrullinated peptides (anti-CCP) were detected by commercially available ELISA for anti-CCP2 (Immunoscan RA; Euro-Diagnostica, Malmoe, Sweden). The results are expressed in U/ml with the cut-off for normal levels at 25 U/ml. Plasma from patients and healthy donors was diluted 100-fold, and ELISAs were performed according to the manufacturers' protocols.

Statistical analysis

Differences in NK cell phenotypes and numbers among patients grouped by disease were analyzed using the nonparametric Mann-Whitney test with a confidence interval of 95%. The significance of NK cell cytotoxicity in the presence or absence of GN8P was evaluated with the paired one-tailed Student's *t*-test. Values of $p\le0.05$ (*), $p\le0.01$ (**), and $p\le0.001$ (***) were considered to be statistically significant.

Results

Reduced NK cell number and effector function in patients with autoimmune diseases

To compare the cytolytic function of NK cells in patients with autoimmune diseases to their function in those with OA and in HD, we examined PBMCs from RA, DM, PM, and OA patients, and from HD using a standard 18-h ^{51}Cr-release assay. There was a highly significant decrease in NK cell cytotoxicity in both RA (32.53%±15.64%, $p<0.0001$) and PM/DM patients (27.81%±14.07%, $p<0.0001$), but not in OA patients (45.61%±19.32%, $p=0.365$) compared to HD (49.76%±15.82%) (Fig. 1A). Thus, NK cell function was impaired only in patients suffering from autoimmune diseases (i.e., RA, PM, and DM).

Because decreased cytotoxicity can be caused by either impaired NK cell function or a lower number of functional NK cells, we correlated our functional assay results with FACS analysis of NK cell percentages. There are two main populations of NK cells that are distinguished by their level of CD56 expression: CD3−CD56high (NK1 type synthesizing TH1 cytokines and NK2 synthesizing TH2 cytokines) and CD3−CD56low (killer-type NK cells); we concentrated on the CD3−CD56low subpopulation. We found a highly significant decrease in the percentage of killer NK cells in PM/DM patients (4.92%±3.0%, $p=0.0006$); however, the NK cell percentage was only slightly decreased in RA patients (6.58%±4.4%, $p=0.003$) compared to HD (8.62%±3.8%) (Fig. 1B). OA patients showed no significant change in the percentage of NK cells (10.39%±3.4%, $p=0.11$). We did not find any significant differences in NK cell activity impairment when comparing different therapeutic interventions or disease stages.

Differences in reactivity to GN8P

Differences in NK cell distribution between RA and HD did not sufficiently explain the highly significant decrease of cytotoxic activity. Therefore, we evaluated the relationship between the impaired function of NK cells and inhibitory activity of CD161 receptor triggered by ligand binding. We used the GN8P glycoconjugate in a 30-min preincubation with PBMC samples as a prototype ligand of CD161 [3] to

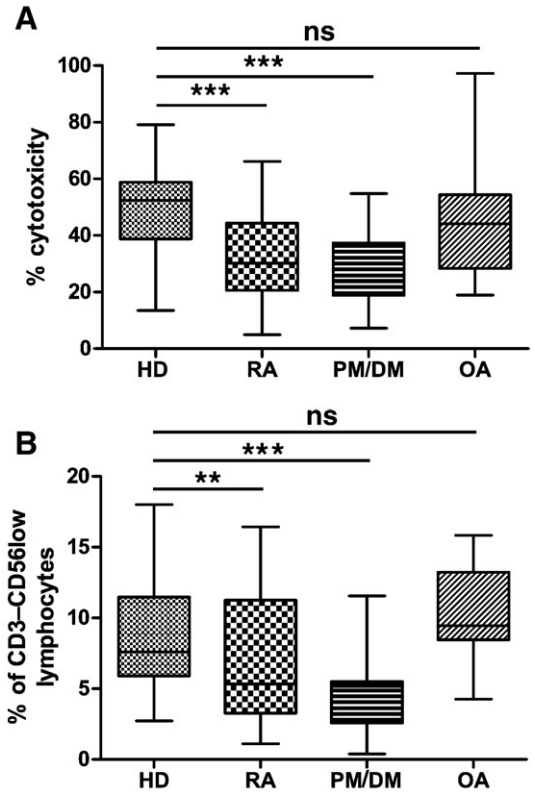

Figure 1 Comparison of NK cell-mediated cytotoxicity and NK cell distribution in the autoimmune diseases RA, PM, and DM with OA and HD. (A) Standard 18-h ^{51}Cr-release cytotoxicity assay was performed with an effector: NK-sensitive K562 target cell ratio of 16:1. Cytotoxicity was significantly impaired in all of the samples from patients with autoimmune disease. OA patient samples had normal cytotoxic activity that was not significantly different from HD. (B) Percentage of NK cells among lymphocytes. The data shown represent the percentage of CD3−CD56low NK cells in patient samples with the same diseases measured in the cytotoxicity assay (**A**). A lower number of killer NK cells corresponded with lower cytotoxicity. Statistical significance according to the Mann−Whitney test is indicated by ** ($p \leq 0.01$), *** ($p \leq 0.001$). ns, not significant ($p > 0.05$). RA $n = 50$, PM $n = 5$, DM $n = 12$, OA $n = 19$, HD $n = 60$.

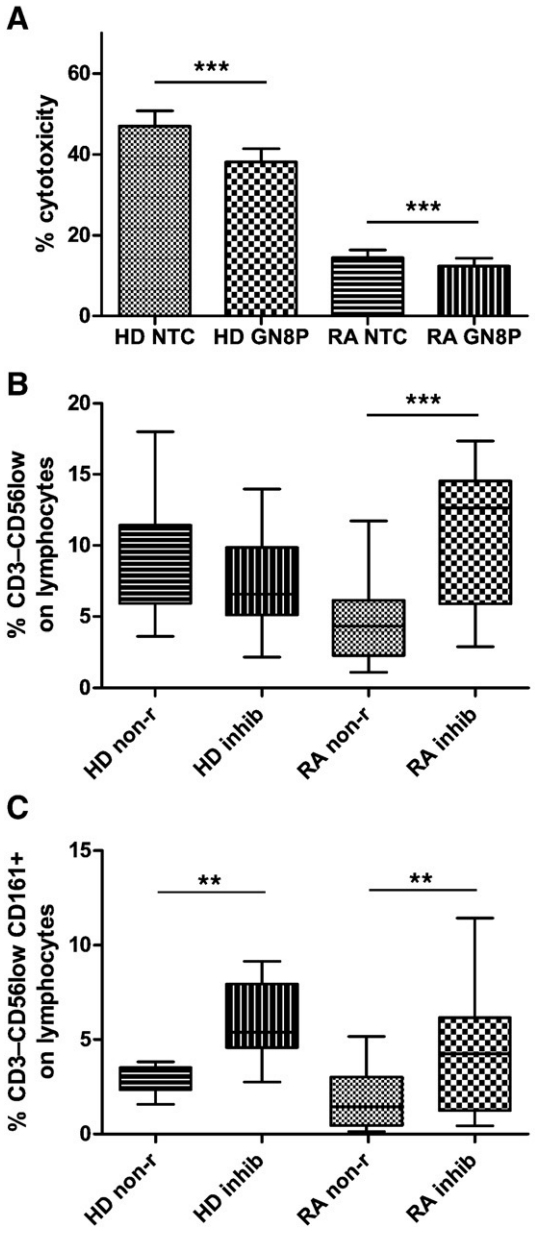

Figure 2 Cytotoxicity, distribution, and phenotype of NK cells in RA patients and HD grouped according to GN8P sensitivity. (A) Cytotoxic activity of GN8P-inhibited samples. NK cell activity measured by 18-h ^{51}Cr-release cytotoxicity assay in the presence or absence (NTC) of GN8P. 50% of RA patient samples were inhibited by GN8P pretreatment, and 50% were GN8P-nonreactive. (B) Percentage of CD3-CD56low NK cells. The plot shows the number of NK cells among lymphocytes divided according GN8P reactivity in HD and RA patients. **C,** Percentage of CD161-positive NK cells (CD3−CD56low CD161+). Significantly higher levels of the CD161+ NK cell subset in GN8P-inhibited samples from both HD and RA patients corresponded with impaired cytotoxicity in the same sample groups. inhib, GN8P-inhibited samples; non-r, GN8P-nonreactive samples. Statistical significance is indicated by ** ($p \leq 0.01$) and *** ($p \leq 0.001$).

investigate how ligand binding affects NK cell functional activity.

We separated the RA patients and HD into GN8P inhibited and non-reactive (data not shown) groups on the basis of differences from control, untreated values. We have taken 5% tolerance, so values under 95% of the untreated controls were taken as inhibited. We found that PBMCs from 50% of the RA patients ($n = 25/50$) *in vitro* responded to GN8P pretreatment by inhibition of NK cell cytotoxicity (Fig. 2A). The mean percentage of cytotoxicity in patients PBMCs not treated with GN8P was 14.44%±8.5%, compared to 12.37%± 7.8% in the GN8P-pretreated PBMCs ($p < 0.0001$). Similarly, in 42% of HD ($n = 26/56$) PBMCs, incubation with GN8P down-modulated the NK cell activity from 46.96%±16.28% to 38.15%±13.8% ($p < 0.0001$), as evaluated by paired, one-tailed Student's *t*-test. However, 50.0% of RA patient PBMCs and 58% of HD PBMCs did not exhibit any significant reaction to GN8P. As we expected, based on what is known about the inhibitory function of the CD161 receptor, the NK cells that

reacted to GN8P showed decreased cytotoxic activity. We found that there was a slightly higher percentage of GN8P-inhibited samples from RA patients compared to HD (50% vs. 42%, respectively). Thus, we next investigated whether the GN8P-mediated decrease in cytotoxicity was caused by a lower number of NK cells, or the inhibitory activity of CD161.

Distribution of NK cells according to reactivity to GN8P

We compared the percentages of NK cells from the two groups of RA patients and HD that were either GN8P inhibited or nonreactive. We found that the percentage of killer NK cells (CD3−CD56low) did not correlate with the difference in cytotoxicity of HD (8.46%±3.7% and 7.44%±3.6%, in nonreactive vs. inhibited, respectively), moreover, in the samples from RA patients, we observed a significant indirect relationship between NK cell percentages and the cytotoxicity assay results: 7.75%±2.8% and 10.98%±4.7% in GN8P-nonreactive and -inhibited samples, respectively ($p=0.0003$) (Fig. 2B). Once we had determined that the percentage of NK cells did not have a direct effect on cytotoxicity as measured by the GN8P assay, we verified the expression of CD161. The total number of CD161-expressing lymphocytes, unlike the percentage of NK cells, correlated indirectly with the cytotoxicity assay results in both HD (15.18%±7.9% vs. 24.92%±7.5% for GN8P-nonreactive and -inhibited samples, respectively; $p=0.005$) and RA patients (12.95%±4.3% vs. 19.80%±5.8% for GN8P-nonreactive and -inhibited samples, respectively; $p=0.0002$). The effect of GN8P on cytotoxicity was likely mediated by binding to CD161 receptors expressed on CD3−CD56low NK cells; ligand binding to CD161 inhibited effector function. Those samples that were inhibited by GN8P had significantly higher percentages of CD161-positive NK cells: HD, 15.18%±7.9% and 24.92%±7.5% in nonreactive and inhibited samples, respectively ($p=0.005$); RA, 1.94%±1.6% and 4.21%±2.1% in nonreactive and inhibited samples, respectively ($p<0.01$) (Fig. 2C).

FACS analysis of CD161-positive lymphocytes showed that RA samples inhibited by GN8P pretreatment even expressed higher levels of CD161 (4300 mean fluorescence intensity [MFI]±304.3 MFI; $p=0.003$) compared to GN8P-inhibited HD samples (2,802 MFI±177.9 MFI). CD161 expression in GN8P-nonreactive HD and RA samples was not significantly different (3,620 MFI±237.3 MFI and 2,991 MFI±224.2MFI, respectively). These findings support the hypothesis that CD161 plays an important role in regulation of NK cell recognition and effector functions.

When we grouped RA patients ($n=50$) according to the therapy that they received (Table 1A), we found that samples from patients receiving either glucocorticoids or immunosuppressives were GN8P inhibited (expressed high levels of CD161): 63% ($n=7/11$) and 66% ($n=6/9$), respectively. In contrast, 70% ($n=14/20$) of the samples from RA patients treated with a combination of glucocorticoids and immunosuppressives were GN8P nonreactive (expressed low levels of CD161). There was no difference in the number of GN8P-nonreactive and -inhibited samples from untreated and TNF antagonist-treated patients (50%). Differentiation of samples from patients according to disease stage revealed that the majority of patients with stage I and II RA fell into the GN8P-inhibited group, while a majority of the samples from patients with stage III and IV RA [31] were nonreactive. All of the samples from patients with active arthritis and inflamed joints (synovial fluid-positive) were GN8P nonreactive (Table 1B).

Table 1 Reactivity of RA patient PBMCs to GN8P according to therapy (A) or disease stage (B).

A			
Therapy	GN8P nonreactive	GN8P inhibited	Total
	Low CD161 expression	High CD161 expression	No. of patients
Glucocorticoids	37%	**63%**	11
Immunosuppresives	34%	66%	9
Glucocorticoids and immunosuppressives	**70%**	30%	20
TNF-antagonists	50%	50%	6
No therapy	25%	**75%**	4
B			
Disease stage	GN8P nonreactive	GN8P inhibited	Total
	Low CD161 expression	High CD161 expression	No. of subjects
I	36%	64%	14
II	44%	56%	9
III	58%	42%	12
IV	60%	40%	10
SF positive	60%	40%	5
Healthy donors	**58%**	**42%**	56

Legend: SF = synovial fluid.

CD161 gene expression in response to GN8P

As NK cells showed major changes in their cytotoxic activity after treatment with GN8P, we wanted to describe this phenomenon in a more detailed manner. We thus investigated the changes that occurred intracellularly following ligand binding by evaluating CD161 – the responsible receptor – expression in purified NK cells. FACS analysis of HD NK cells revealed that untreated HD NK cells fell into two groups: the first group had high numbers of NK cells expressing CD161 receptor, and the second group had low numbers of NK cells expressing CD161 receptor (Fig. 3A).

NK cell reactivity to GN8P correlated with the cytotoxic response of the donor cells. In response to GN8P, subjects with higher numbers of NK cells (CD56+CD3−) expressing CD161 protein showed a slight reduction in CD161 mRNA expression, as measured by RT-PCR. However, the donors with low numbers of CD161+ NK cells resulted in a slight increase in CD161 mRNA expression (Fig. 3B).

MGAT5 gene expression in response GN8P

The role of the glycosyltransferase MGAT5, a key glycan metabolism enzyme, in autoimmune disease was previously

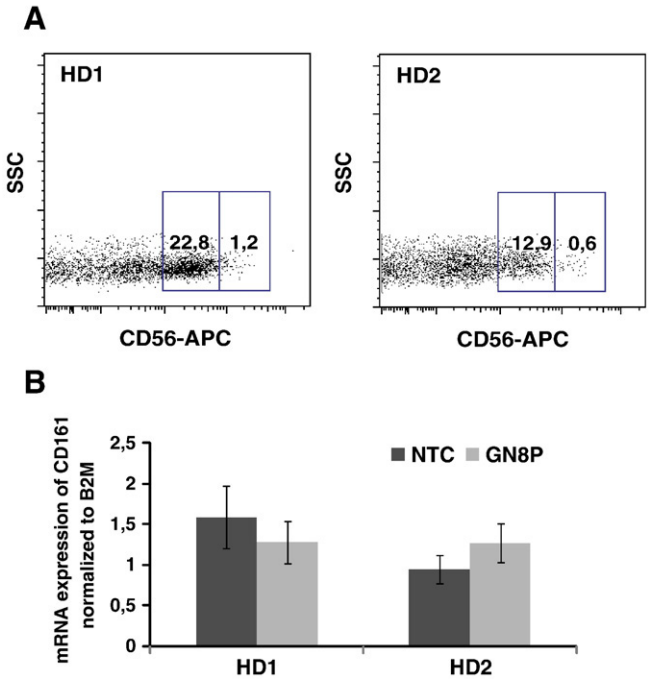

Figure 3 CD161 mRNA expression by purified NK cells incubated with GN8P. (A) Distribution of cell populations (percentage) in representative HD with a higher percentage of CD3−CD56low NK cells (HD1) and a lower proportion of CD3−CD56low NK (HD2). The dotplots show CD56/SSC gated on CD161 positive cells. (B) CD161 mRNA expression detected by RT-PCR and normalized to control gene β-2-microglobulin (B2M). Purified NK cells (CD56+CD3−) were incubated in the presence or absence (NTC) of GN8P. Two different patterns were identified in response to GN8P: a group ($n=6$) in which CD161 mRNA expression was inhibited by GN8P (HD1), and a group ($n=7$) that did not exhibit GN8P-mediated inhibition (HD2).

studied in a mouse model. Demetriou et al. [14] have found that a deficiency in MGAT5 lowers T-cell activation thresholds by directly enhancing TCR clustering. MGAT-5-deficient mice showed kidney autoimmunity as well as an increased susceptibility to experimental autoimmune encephalomyelitis. We have thus wanted to elucidate the role of MGAT5 under the conditions of human RA. To determine if MGAT5 expression differs between PBMCs from RA patients and HD, or between PBMCs and synoviocytes from RA patients, we performed real-time RT-PCR. PBMCs from HD expressed lower levels of MGAT5 mRNA than did PBMCs from RA patients. Synoviocytes, the adherent cell population within synovial fluid extracted from the joints of RA patients, expressed significantly higher levels of MGAT5 than did PBMCs from RA patients.

Because GlcNAc compounds similar to synthetic GN8P are involved in modulation of the glycan profile in RA, we investigated whether GN8P modulated the expression of MGAT5 mRNA. GN8P significantly downregulated the expression of MGAT5 mRNA in synoviocytes ($p=0.05$). However, GN8P did not affect MGAT5 mRNA expression in either the HD or the RA patient samples ($p=0.35$ and $p=0.5$, respectively) (Fig. 4A and B).

CD161 and PAD4 gene expression in response to MCV autoantigen

We next evaluated changes in the CD161 and PAD4 mRNA expressions of purified NK cells from RA patients or HD in response to incubation with either the RA-specific autoantigen MCV or non-citrullinated vimentin (VIM, data not shown). NK cells from HD expressed significantly less CD161 mRNA than NK cells from RA patients ($p=0.0171$). Incubation of HD NK with VIM or MCV led to a significant increase in CD161

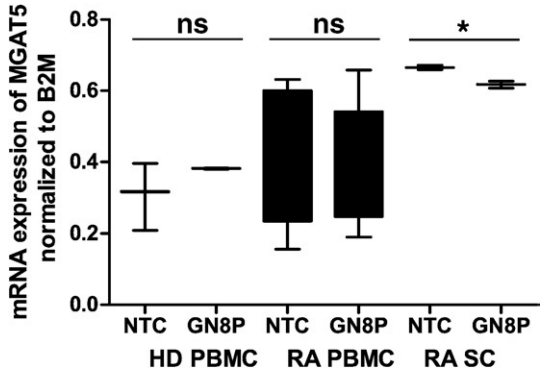

Figure 4 MGAT5 mRNA expression in PBMCs and synoviocytes. MGAT5 mRNA expression in PBMCs from HD (HD PBMC), RA patients (RA PBMC), and synoviocytes extracted from the synovial fluid of RA patients (RA SC). Cells were incubated in the presence or absence (NTC) of GN8P and gene expression was detected by real-time RT-PCR represented as the mean ± max/min values of 5–7 samples. β-2-microglobulin (B2M) was used for PCR normalization. Synoviocytes from RA patients expressed high levels of MGAT5 mRNA, and incubation with GN8P led to a decrease in MGAT5 mRNA expression. Statistical significance is indicated by * ($p \leq 0.05$). ns, not significant ($p > 0.05$).

mRNA expression ($p=0.0083$ and 0.0088, respectively). NK cells from RA patients did not exhibit a similar increase in CD161 mRNA expression following incubation with MCV ($p=0.2$) (Fig. 5A). Because HD NK cells responded to MCV or VIM treatment but RA NK cells did not, we next investigated whether there were differences in the expression of PAD4, the enzyme responsible for citrullination, in RA patient and HD NK cells, and whether incubation with MCV or VIM affected PAD4 expression levels. Equal levels of PAD4 mRNA were expressed in purified NK cells from HD and RA patients ($p=0.41$). Incubation of HD NK cells with VIM or MCV resulted in a significant increase in PAD4 mRNA ($p=0.0083$ and 0.0025, respectively). This upregulation in PAD4 expression did not occur when NK cells from RA patients were incubated with MCV ($p=0.5$) (Fig. 5B).

To determine the level of disease activity in RA patients that were used in mRNA analysis, we tested for the presence of anti-MCV and anti-CCP antibodies. As shown in Fig. 5C, all of the RA samples contained high levels of anti-MCV and anti-CCP antibodies. HD and OA patients (non-RA) had antibody levels that were within normal limits (Fig. 5C).

Discussion

NK cells participate in preserving immunological tolerance by providing negative feedback to activated macrophages as well by influencing other cell types. NK cells can suppress autoreactive B or T lymphocytes, which may explain the protective role of NK cells in some autoimmune diseases [38,39].

We found that NK cell-mediated cytotoxicity was impaired in RA and PM/DM patients in contrast to OA patients and HD. In contrast to PM/DM patients, who exhibited both decreased cytotoxicity and lower NK cell numbers, the percentage of killer NK cells in RA patient samples did not correlate well with decreased cytotoxicity levels. Thus, we further explored the role of NK cells in patients with RA.

Because a previous study proposed that glycosylation plays an important role in the pathogenesis of autoimmunity [1], we focused on expression of the lectin receptor CD161, which recognizes and binds glycoproteins with GlcNAc moieties [3]. We used GN8P as a prototype ligand to determine how glycan recognition inhibits NK cell function in RA patients and HD. We divided the patients into GN8P-inhibited and -nonreactive groups depending upon how NK cell cytotoxicity responded to incubation with GN8P. We believe that the inhibitory effect was caused by ligand binding to CD161, which is in agreement with the findings of Rosen et al. [4]. This hypothesis was supported by the results of FACS analysis, which showed a correlation between increased CD161 expression on NK cells and decreased cytotoxicity. Upregulation of CD161 mRNA expression in purified NK cells after ligand induction was confirmed by RT-PCR (Fig. 3).

The differences between CD161 low and high expressers according to the disease stage correlate with the seriousness of the illness that corresponded as well with the therapeutic protocols (Table 1A, B). Majority of the patients with severe stages of illness undergo more powerful therapeutic procedures, either combination of glucocorticoids and immunosuppressant or TNF antagonist treatment. Further possible explanation of higher proportion of GN8P nonresponsive NK cells in patients with the exacerbation of arthritic symptoms could be their recruitment into the joint microenvironment and their consequent absence in peripheral blood.

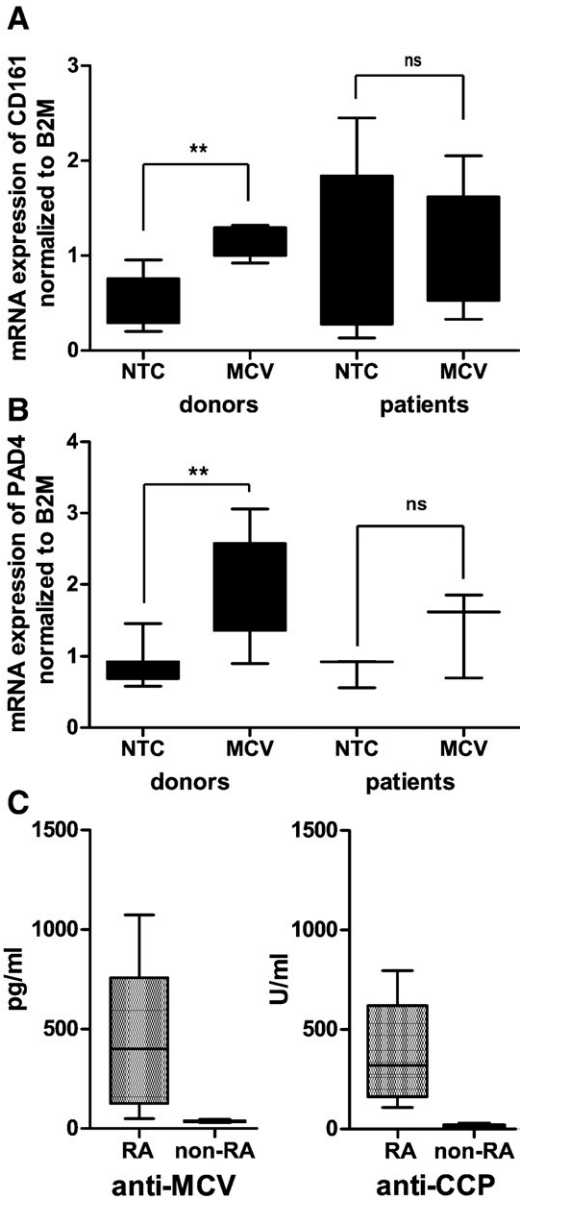

Figure 5 CD161 and PAD4 mRNA expression in NK cells from HD and RA patients. CD161 (A) and PAD4 (B) mRNA expression detected by RT-PCR and normalized to the control gene β-2-microglobulin (B2M). Purified NK cells were incubated in the presence or absence of MCV. Nontreated controls (NTC) were cells incubated without MCV. Statistical significance is indicated by ** ($p \leq 0.01$). ns, not significant ($p > 0.05$). (C) Determination of anti-MCV and anti-CCP antibodies in plasma samples from RA patients and HD/OA selected for mRNA analyses (A, B). RA, patients with active disease; non-RA, HD and OA patients. Plasma samples from RA patients had high levels of anti-MCV and anti-CCP antibodies in contrast to healthy donors. NK cells from healthy donors, but not RA patients, responded to incubation with MCV by increased expression of CD161 and PAD4 mRNA.

In the active disease 2/3 of patients fall into the group GN8P inhibited, CD161 high expressing NK cells that supports the hypothesis about the importance of CD161 molecule in the course of RA and the sensitivity of patients to the inhibitory action of glycans produced on the surface of synoviocytes [9,10,14].

From the therapeutic point of view the best results were achieved by the combined treatment with glucocorticoids and immunosuppressive drugs (70%) followed by TNF antagonist treatment (50%). The single glucocorticoid or immunosuppressive therapy was less effective, 37% and 34%, respectively, as depicted in the Table 1A.

We found that CD161 expression was increased not only on NK cells, but also on other type of lymphocytes from RA patients, including T and NKT cells, which, when activated, could deepen the severity of the disease (data not shown). Exley et al. [40] showed that CD161 can act as a specific costimulatory molecule for TCR-mediated proliferation and cytokine secretion by $V_{\alpha}24^{invt}$ T cells. Ligation of CD161 in the absence of TCR stimulation does not result in $V_{\alpha}24^{invt}$ T cell activation, and costimulation through CD161 does not cause polarization of the cytokine secretion pattern. However, CD161 signaling events remain unclear.

A number of enzymes that synthesize N- and O-linked oligosaccharides are active in synoviocytes ([41], for review see [42]). To explore the molecular events underlying glycosylation pathways in the synovium of RA patients, we examined the effect of GN8P on expression of the glycosyltransferase MGAT5. The synthetic glycoconjugate GN8P can serve as a substrate for MGAT5, which is responsible for GlcNAc branching. GlcNAc branching plays a key role in NK cell recognition in autoimmune conditions [43–45]. We found that MGAT5 was involved in GlcNAc metabolism in the synoviocytes of RA patients with active disease, but not in their PBMCs. Metabolic regulation of GlcNAc branching on synoviocytes, but not on peripheral blood immune cells, may indicate genetic predisposition to RA susceptibility. GlcNAc-terminated glycans may be involved in upregulation of CD161 expression, resulting in downmodulation of NK cell function in patients with RA. Similarly, GN8P, acting as a substrate for synoviocytes, may reduce MGAT5 mRNA expression.

A characteristic feature of RA is the reaction of lymphocytes to the autoantigens MCV and VIM. We tested the effect of human MCV and recombinant VIM on CD161 expression in NK cells derived from HD and RA patients. Both MCV and VIM led to a significant increase in CD161 mRNA levels in NK cells from HD. In RA patients, where MCV and VIM expression is elevated; we found that NK cells did not exhibit any further change in CD161 expression levels when incubated with MCV or VIM in vitro. Thus, the increased expression of CD161 seen in RA patient NK cells may be due to continual stimulation by citrullinated VIM or to higher levels of free VIM from damaged cells.

In this study, we showed, to our knowledge for the first time, that in addition to macrophages and granulocytes, the main producers of PAD4 enzyme [25], NK cells are also able to activate the PAD4 in response to VIM or MCV. Incubation of HD NK cells with either VIM or MCV led to upregulation of PAD4 mRNA. This was not observed in NK cells derived from RA patients, which had already been exposed to the MCV autoantigen. Thus, we deduce that NK cells may be involved in the citrullination of vimentin in patients with RA.

Conclusions

We discovered that NK cells of healthy donors recognize the RA autoantigen MCV. Exposure to MCV may serve as the triggering mechanism for induction of CD161, as well as increased expression of the citrullination enzyme PAD4. We also provide evidence, for the first time to our knowledge, that high levels of CD161 receptor on NK cells are correlated with impairment of NK cytotoxic activity in RA patients. We propose that MCV plays an important role in RA by increasing expression of CD161, which leads to inhibition of NK cell activity and, consequently, insufficient downregulation of autoreactive T cells, thus accelerating disease progression.

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3.2 Glycosylation regulates NK cell-mediated effector function through PI3K pathway

3.2.1 Overview

Aberrant glycosylation profile is closely associated with improper cell function in diseases like cancer [188]. These changes are based on loss of certain structures or, on the contrary, their overexpression [189]. MGAT3 and MGAT5 are two crucial glycosyltransferases responsible for linking N-acetyl-D-glucosamine (GlcNAc) residues on glycans. MGAT5 forms β1-6 branched structures that are particularly abundant in aberrant cancer cell glycosylation profile. MGAT3, on the other hand, catalyses the addition of β1-4 residues and forms structures that further block the addition of other GlcNAc residues. Although the enzymes compete with each other during the glycosylation process leading to the suppression of metastatic growth, they are both overexpressed in tumour cells [190-192].

NK cells were reported to express receptors recognizing carbohydrate structures such as sialyl Lewis x on cells as cytolytic signals or stimulating signals for cytokine production [193]. GN4C is a synthetic glycodendrimer bearing 4 GlcNAc moieties on calix[4]arene core. As saccharides generally have low binding affinity to its receptors, the multivalent nature of the dendrimer is a beneficial feature making it good prototype structure for studying cell reaction to carbohydrates. In this study, we used GN4C glycodendrimer as a prototype ligand for studying the reaction of NK cells to glycan structures. The experiments were performed on NK-92 cell line as well as fresh human NK cells.

3.2.2 Aims and methods

The aim of this study was to provide a comprehensive analysis of NK cell reaction to GlcNAc-bearing structures in terms of signalling, function and induction of glycosylation-related processes. NK-92 cell line and sorted fresh human NK cells were used in the study. We used glyco-gene profiling array to monitor the expression of glycosylation-relevant genes. PCR-based methods were used for the detection of MGAT3 and MGAT5 glycosyltransferases as well as surface NK receptors and cytokines. We used 3H-thymidine proliferation and ^{51}Cr-release cytotoxicity assays to monitor the functional parameters of the cells. Flow cytometry and CBA cytokine array was used to detect the cytokine profile and protein phosphorylation pattern was detected by Prosphotyrosine profiling array and immunoblot.

3.2.3 Results and discussion

GlcNAc-bearing glycoconjugates were previously used in tumour-bearing animals with significant effects on tumour growth accompanied by enhanced immune response [194, 195]. In this study, we analysed the effect of GN4C on the expression of relevant glycosyltransferases MGAT3 and MGAT5. We showed that GN4C interferes

with the glycosylation processes by downmodulating the expression of both enzymes which was proved in NK-92 cell line as well as in fresh NK cells. Another significant result is the upregulation of NK cytotoxicity upon GN4C treatment again in both NK-92 and fresh NK cells. This functional change corresponds with the upregulation of the expression of NKG2D activating receptor. The exact receptor responsible for binding GN4C is, however, unknown.

In this paper, we did not analyse only the direct reaction of NK cells to the glycodendrimer but also the reaction of untreated NK cells to GN4C-pretreated tumour cells as these carry a number of glycosylated structures and may also be affected by the GN4C influence on glycosylation pathways. We used HT-29 colorectal carcinoma and K562 chronic myeloid leukaemia cells as targets of NK cell-mediated lysis. GN4C treatment made HT-29 cells more sensitive to NK-mediated lysis by both fresh NK cells and NK-92. As NK cells are targeted preferentially against NK-sensitive hematopoietic cells, the increased susceptibility of the colorectal carcinoma cells is an important result [196]. Using the same approach to K562 leukaemia cells, we found the expected increase only using fresh NK cells as effectors, while NK-92 cells showed an opposite trend. This may be caused by K562 cells expressing a different glycan pattern, so that triggering the glycosylation pathways by GN4C may lead to an opposite effect, masking ligands for NK-92 recognition.

Another important result is the change in cytokine production after GN4C treatment stimulating particularly Th1 cytokines. In NK-92 cells, intracellular FACS analysis as well as RT-PCR and CBA showed an increase in TNF-α, IFN-γ and IL-2. In fresh NK cells, these changes were not so prominent with the most important change being the IL-2 increase. Because human NK cells require IL-2 to trigger their antitumour cytotoxic activity, the observed IL-2 increase may induce the necessary autocrine stimulation [197].

Regarding the signalling experiments, GN4C triggered the PI3K/ERK1/2 pathway which is in agreement with data published earlier [198]. This was also proved by the use of PI3K inhibitor wortmannin that abolished GN4C-mediated cytotoxicity in both NK-92 and fresh NK cells. Regarding the possible involvement of PLC-γ signalling pathway, phosphorylation status in western blot analysis did not show significant difference, so we could rule this pathway out.

In conclusion, GN4C was found to be a potent modulator of NK cell function both directly and indirectly (response to GN4C-affected tumour cells). Its main function lies in the modulation of MGAT3 and MGAT5 glycosyltransferases leading to improvement of NK effector function and augmentation of tumour sensitivity to NK-mediated cytotoxicity. These results demonstrate the potential of GN4C as a modulator of NK cell response under cancer, but possibly also other, pathological conditions.

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Glycosylation regulates NK cell-mediated effector function through PI3K pathway

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Abstract

Aberrant glycosylation, which impairs recognition capability of NK cells or modifies recognition pattern of target cells, is associated with cancer. Synthetic glycoconjugates (GCs), which modulate cell glycosylation, increase the sensitivity of tumor cells to therapy or boost anti-cancer immune response. In the current study, we employed *N*-acetyl-D-glucosamine-calix[4]arene (GN4C) as a modulator of cell glycosylation of NK cells represented by the NK-92 cell line and fresh human NK cells. For the first time, we have demonstrated that calix[4]arene-based GC down-regulated the expression of glycosyltransferases *MGAT3* and *MGAT5* in NK-92 and fresh NK cells. GN4C increased the susceptibility of tumor cells to cytotoxicity by purified fresh NK cells or NK-92 cells. This functional activation of NK cells and the NK-92 cell line correlated with an increased expression of *NKG2D* mRNA. In the NK-92 cell line, GN4C induced the synthesis of IL-2, IFN-γ and tumor necrosis factor-α as well. Cellular signaling triggered by GN4C engaged PI3-kinase/ERK but not phospholipase C-gamma/JNK pathways. Simultaneously, in transformed NK-92 cells, GN4C reduced the rate of proliferation and down-regulated the *c-MYC*, *EGF-receptor 1* and REL-A molecules. In conclusion, the modulation of glycosyltransferases *MGAT3* and *MGAT5* by synthetic GN4C correlated with the improvement of NK cell effector functions and the augmentation of tumor cells sensitivity to NK cell-mediated cytotoxicity.

Keywords: cytotoxic activity, gene regulation, glycoconjugate, glycosyltransferases, NK cells, NKG2D, NK-92

Introduction

A particular glycan profile on the cell surface is crucial for proper cell function, and aberrant glycosylation is associated with diseases like cancer (1). Changes in glycosylation arise from alterations in the expression level of glycosyltransferases.

N-acetyl-D-glucosamine (GlcNAc) transferases MGAT3 and MGAT5 have major involvement in linking terminating residues on glycans. MGAT5 is responsible for adding β1-6 GlcNAc residues and forming branched structures, which are especially abundant in cancer tissues with high metastatic potential. MGAT3 catalyzes the addition of β1-4 GlcNAc residues and forms a bisecting structure that disables further addition of GlcNAc by other glycosyltransferases like MGAT5. Competition of MGAT3 and MGAT5 *in vivo* leads to the suppression of cancer metastasis (2). Nevertheless, both enzymes, MGAT3 and MGAT5 tend to be over-expressed in tumor cells (3, 4).

NK cells recognize particular carbohydrate structure as cytolytic signals (5), triggered by inhibitory and activation receptors on the cell surface regulating NK cell-mediated cytotoxicity and production of chemokines and inflammatory cytokines (6).

NKG2D is a functional activation receptor of NK-92 cells, which plays a role in NK cell-mediated tumor recognition and cytolysis (7). Stimulation of NKG2D recruits two critical signaling components, the regulatory subunit p85 of PI3K and phospholipase C-γ (PLC-γ), which results in elevated levels of cytokines tumor necrosis factor (TNF)-α and IFN-γ as well as degranulation and cytotoxicity (8). Cross linking of NK cell receptors like NKG2D results in activation of kinases ERK and JNK. Activation of ERK and JNK triggers the microtubule-organizing center and granule polarization in NKG2D-mediated NK cell cytotoxicity (9). NKR-P1A (CD161) is a representative of human inhibitory receptors. Signaling through this receptor and its function remains unclear. Oligosaccharides such as *N*-acetyl-D-glucosamine serve as ligands for recombinant NK cell receptor NKR-P1A in rats (10).

The NK-92 tumor cell line is a widely used model for NK cell studies. We used those cells to characterize the effect of glycoconjugate (GC) *N*-acetyl-D-glucosamine-calix[4]arene (GN4C) on human NK cell functions. Nevertheless, NK-92 cells were derived from a malignant non-Hodgkin's lymphoma; thus, it gives also insight of the effect of GN4C on tumor cells. The NK cell functions of the NK-92 cell line were confirmed by the use of fresh NK cells obtained from healthy donors.

We have recently reported that GC consisting of four GlcNAc residues on calix[4]arene core (GN4C) promotes cell-mediated cytotoxicity of human PBMC against K562 target cells (11). This GC exhibits affinity to rat NK cell receptor; thus, we investigated the effect of GN4C on human fresh NK cells and the NK-92 cell line, including gene expression changes and NK cell effector functions. We determined GN4C-mediated modulation of glycosyltransferases, proliferation factors and NK-specific molecules and functions. We showed that GN4C increases susceptibility of tumor cells to NK cell-mediated cytolysis. This study provides evidence of the complex effect of GN4C on NK-92 cells and determines the NK portion of output results.

Methods

Preparation and treatment of cells with GC

The NK-92 cell line (NK cell leukemia) was obtained from American Type Culture Collection (ATCC) (CRL-2407) and maintained in α-MEM medium containing 100 U ml^{-1} of IL-2 as described previously (12). HT-29 (colon carcinoma) and K562 (chronic myeloid leukemia) cell lines were obtained from ATCC (HTB-38 and CCL-243) and maintained in RPMI-1640 medium with 10% FCS. Before the experiment, NK-92 cells starved of IL-2 for 18 h. Purified human NK cells (>98%) were isolated from peripheral blood of healthy donors (peripheral blood mononuclear cells, PBMC) using Ficoll-Paque density gradient (1.077) and CD56+CD3− cell population was sorted with a FACS Vantage SE (BD Bioscience, Heidelberg, Germany). Within PBMC samples, distinct populations were determined with the BD LSRII (BD Bioscience) using multicolor analysis employing antibodies CD3-Pacific Blue (Dako, Glostrup, Denmark), CD4-APC-Alexa 750 (Caltag, Burlingame, CA, USA), CD8-Pacific Orange (Caltag), CD56-APC (Exbio, Prague, Czech Republic) and CD56-PE (Immunotech, Marseille, France). GC calix[4]arene with four terminal *N*-acetyl-β-D-glucosamine moieties (GN4C) which is solubilized in cell culture media was synthesized and kindly provided by Karel Krenek (Academy of Sciences of the Czech Republic, Prague, Czech Republic). Synthesis, purity, NMR data and dose-dependent effect of this GC on human PBMC were described previously (11), and the optimal concentration of 10 nM was used for our experiments.

Glyco-gene profiling array

The NK-92 cell line was incubated with GN4C for 24 h and total RNA was isolated with RNAeasy Mini Kit involving DNAse I treatment as described by the manufacturer (Qiagen, Hilden, Germany). A custom-designed Glyco-gene Chip array (GLYCOv3 Gene Chip) that contains probe sets to monitor the expression of ~2000 human transcripts relevant to the Consortium for Functional Glycomics has been developed using Affymetrix technology. The microarray experiments (triplicates of each sample) were performed by The Microarray Gene Core of Consortium for Functional Glycomics, NIH/NIGMS (http://www.functionalglycomics.org). BRB ArrayTools [Biometric Research Branch, NIH/National Cancer Institute (NCI), Bethesda, MD, USA] were used to filter and analyze experimental data sets. Class comparison used a two-sample *t*-test with a random variance model, and differences with *P*-value of log-ratio <0.05 were considered significant. Gene Ontology analysis was performed using DAVID software available from NCI (http://david.abcc.ncifcrf.gov).

Reverse transcriptase–PCR and real-time reverse transcriptase–PCR

Total RNA from the NK-92 cell line or fresh NK cells incubated with GN4C for 24 h was used for semi-quantitative expressional analysis by real-time reverse transcriptase (RT)–PCR as well. Five micrograms of RNA were transcribed into cDNA using cDNA Archive Kit (Applied Biosystem, Foster City, CA, USA). RT–PCR for detection of cytokine mRNA, epidermal growth factor receptor 1 (*EGFR1*) and *Ki-67* was carried out with HotStarTaq DNA Polymerase (Qiagen) and an iCycler5 (Bio-Rad, Philadelphia, PA, USA). Real-time RT–PCR was performed with PowerSybr® Green Master Mix (Applied Biosystem) and an iCycler5. PCR product specifity was checked by melt curve analysis. The primers designed by us with the Primer3 Input software were as follows: *MGAT5*: F (forward)—5′-CTTCTTTCTTCCAGCACCTCAAC-3′ and R (reverse)—5′-AAACACACAGTGCTTATTCTTAGGG-3′, *NKG2D*: F—5′-CACAGCTGGGAGATGAGTGA-3′ and R—5′-CTAC AGCGATGAAGCAGCAG-3′, *NKR-P1A*: F—5′-TCTTCCTCGG GATGTCTGTC-3′ and R—5′-CCTGCTCTGTTGAATGTCCA-3′. The sequences of the primers used to amplify β-2-microglobulin (*B2M*), *EGFR1*, *c-MYC*, *Ki-67*, *IFN-γ* and *TNF-α* were described previously (13, 14–17). The primers for *MGAT3* gene detection (PPH01058A) were obtained from SA Biosciences (Frederick, MD, USA). The gene of interest was normalized to the control gene and differences in gene expression between untreated and GN4C-treated cells were evaluated with Bio-Rad iQ5 2.0 software.

^{3}H-thymidine proliferation assay

The NK 92-cell line (1.25×10^3, 2.5×10^3, 5×10^3 200 μl^{-1} per well) and fresh human NK cells (2.5×10^4 200 μl^{-1} per well) were plated in tetraplicates on 96-well plates and cultured in presence of GN4C (10 nM) or IL-2 (100 U ml^{-1}) for 1–3 days. Cells cultured at the same density in the absence of IL-2 or GN4C were used as controls. At the end of the time period, 18.5 kBq per well of 3H-thymidine (GE Healthcare Life Sciences, Amersham, UK) was added to each well and samples were subsequently harvested after 8 h and analyzed as described earlier (11).

Cytotoxicity assay

NK cell-mediated cytotoxicity was performed as described previously (18). The cell lines and fresh NK cells were incubated with or without GN4C (1 dose, 10 nM) for 30 min. In experiments with a PI3K inhibitor, NK-92 cells and fresh NK cells were incubated with wortmannin (50 nM, Calbiochem,

Darmstadt, Germany) for 30 min and subsequently with or without GN4C. The NK-92 cell line and fresh NK cells with or without GN4C pre-treatment were used as effectors. HT-29 and K562 cell lines with or without GN4C pre-treatment were used as targets for evaluation of cell-mediated cytotoxicity. The effector:target ratios were optimized for the experiment at 3.5:1 for NK-92 cells and 10:1 for NK cells. Release of ^{51}Cr was measured after 18 h of incubation.

Cytokine detection by flow cytometry

For intracellular detection of cytokines, the NK-92 cell line, PBMC and purified NK cells were incubated with GN4C for 30 min, fixed and permeabilized with BD Cytofix/Cytoperm™ according to the manufacturer's protocol (BD Bioscience). TNF-α and IFN-γ were detected with anti-human TNF-α-APC (Mab11, eBioscience, San Diego, CA, USA) and IFN-γ-APC monoclonal antibodies (Caltag, Buckingham, UK), respectively. The isotype controls IgG_1 (BD Bioscience) and IgG_{2b} (BD Bioscience) were used to determine the non-specific fluorescence of TNF-α and IFN-γ, respectively.

To confirm the results and broaden the panel of tested cytokines, we used BD™ Cytometric Bead Array Human T_h1/T_h2 (BD Bioscience). This array consisted of six cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ), which were detected in cell supernatants as well as in cell lysates prepared as described previously (19). The production of cytokines was measured with a flow cytometry BD LSRII and analyzed with FlowJo 7.2.2 software.

Detection of protein phosphorylation pattern

To evaluate tyrosine phosphorylation, the NK-92 cell line was incubated with GN4C for 10 min and immediately placed on ice. Cell lyses and detection of SH2 domains, binding specific phosphorylated kinases, was performed with the TranSignal™ Phosphotyrosine Profiling Array (Panomics, Heidelberg, Germany) according to the manufacturer's protocol. This array includes 38 different human SH2 domains in duplicates that bind to their targeted tyrosine phosphorylated proteins. Membranes were scanned with a Fujifilm LAS-1000 (Berthold, Bundoora, Australia) and normalized to equal intensity, and spot densities were evaluated with ImageJ (NIH) software.

Phosphorylation of signaling molecules was confirmed by immunoblot of total protein lysate from NK-92 or sorted NK cells. Protein lysate preparation and immunoblot were described previously (19). Phosphorylation of PLC-γ (Tyr783), ERK1/2 (p44/42 MAPK, Thr202/Tyr204), JNK1/2 (SAPK/JNK, Thr183/Tyr185) and REL-A (NF-κB p65) were detected with the antibodies obtained from Cell Signaling Technology (Danvers, MA, USA). mAb anti-PI3K (p85α) was obtained from Millipore (Billerica, MA, USA). The membranes were reprobed with a mouse mAb to GAPDH (Chemicon, Tamacula, CA, USA) as a control for sample loading. Immune complexes were detected by incubation with peroxidase-conjugated anti-mouse (Jackson ImmunoResearch, West grove, PA, USA) or anti-rabbit (Zymed, San Francisco, CA, USA) secondary antibodies and SuperSignal® West Pico Chemiluminiscent Substrate Kit (Thermo Scientific, Rockford, IL, USA). Blots were subjected to densitometric analysis as described above. 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, Sigma–Aldrich, Taufkirchen, Germany) served as a positive inducer of MAPK phosphorylation. In experiments with PI3K inhibitor, NK-92 cells and fresh NK cells were incubated with wortmannin (50 nM) for 30 min and subsequently with or without GN4C.

Statistical analysis

Statistically significant differences in the parameters tested in NK-92 cells or fresh NK cells cultured in the presence or absence of GN4C were assessed using the non-parametric Mann–Whitney test with a confidence interval of 95%. Values of $P \leqslant 0.05$ (*), $P \leqslant 0.01$ (**), $P \leqslant 0.001$ (***) were considered to be statistically significant.

Results

mRNA expression changes in NK-92 cells involved genes related to cancer progression

Particular surface glycosylation is essential for NK cell functioning. We employed transformed cell line NK-92 to evaluate the effect of GN4C on glycosylation-related genes in NK cells. To determine gene expression changes caused by GN4C, we undertook a complex approach analyzing the glyco-gene expression profiling. To identify genes differentially expressed in NK-92 cells, in the presence or absence of GN4C, we used high-density oligonucleotide microarray. We excluded (with BRB Array Gene Filter) all genes in which the percentage of absent data exceeded 50% and *P*-value was >0.05. Class comparison revealed 24 genes displaying significantly (2-fold change, *P*-value < 0.05) different expressions between control and GN4C-treated cells. The GN4C-responsive genes were functionally categorized according to Gene Ontology classification and then mapped to biological pathways with the use of the KEEG and BioCarta Pathways. The listed genes encoded glycoproteins with N-linked glycosylation site (GlcNAc) (46% of filtered genes), growth factors (17%) and mitogens (13%). According to the functional classification, GN4C influenced transfer of hexosyl/glycosyl groups (38% of filtered genes is involved), signal transduction (38%), carbohydrate binding (25%), regulation of cell proliferation (25%) and organ development (25%). Cellular response to GN4C involved glycan biosynthesis, MAPK, JAK-STAT and transforming growth factor (TGF)-β signaling pathways. Considering disease relationship, 77% of the genes listed were linked to cancer (Gene Card database). A complete list of those differentially expressed genes related to cancer is shown in Table 1.

GN4C GC effected mRNA expression of glycoproteins and enzymes involved in their processing the most. Along with other genes, GN4C-mediated down-regulation of glycosyltransferases *MGAT3* and *MGAT4B*, *JUND*, *TGF-β1* and vascular endothelial growth factor B (*VEGF-B*). These genes play an important role in cancer progression, and inhibition of their expression is of particular interest. Up-regulated genes included galactosaminyltransferase *GalNT4*, *Siglec-8* and Sialomucin. Groups of glycosyltransferases and positive regulators of proliferation were further examined by real-time RT–PCR.

Table 1. Output list of GN4C-responsive genes related to cancer in the NK-92 cell line (determined by online databases Gene Ontology and Gene Cards)

Gene	Description	GI	Biological function
Up-regulated genes			
CD164	Sialomucin	34222157	GP, ST, RCP, OD, I
Down-regulated genes			
CAPN1	Calpain 1	12408655	RCP, OD, apoptosis
CHRD	Chordin	11494372	GP, GF, CB
EPOR	Erythropoietin receptor	182200	GP, ST
FLT3LG	fms-related tyrosine kinase 3 ligand	494978	GP, ST, RCP, OD, I
FLNA	Filamin A	160420313	ST, actin filament binding
JUND	Jun D proto-oncogene	5177963	TF
MDK	Midkine	182650	M, T, ST, CB, RCP, OD
MGAT3	Beta-1,4-*N*-acetyl-glucosaminyltransferase	148539887	T
MGAT4B	Beta-1,4-*N*-acetyl-glucosaminyltransferase	11282	T
MST1	Macrophage stimulating 1	31543211	GP, GF
TGFB1	Transforming growth factor beta 1	12652748	GP, GF, M, ST, TF, RCP, OD, I
VEGF-B	Vascular endothelial growth factor B	39725673	GP, GF, M, ST, T, CB, RCP, neovascularization

CB, carbohydrate binding; GF, growth factor; GI, database accession number; GP, glycoprotein; I, immune process; M, mitogen; OD, organ development; RCP, regulation of cell proliferation; ST, signal transduction; T, transferase; TF, transcription factor.

Glycosyltransferases and cell growth regulators displayed decrease of gene expression

For deeper examination of GN4C effect on glycosylation machinery, we examined mRNA expression of glycosyltransferases, involved in terminal glycan elongation. Housekeeping gene for *B2M* exhibited stable expression in our experimental system and was further used as control gene for data normalization. In fresh NK cells as well as in the NK-92 cell line, GN4C down-regulated the expression of both glycosyltransferases *MGAT3* (P = 0.0001 and 0.0316) and *MGAT5* (P = 0.0001 and 0.0467) (Fig. 1). Down-regulation of *MGAT3* expression in NK-92 cells detected by real-time RT-PCR agreed with the equal result of *MGAT3* expression obtained by glyco-array described above.

The NK-92 is a transformed tumor cell line; thus, we examined if GN4C affected proliferation factors *c-MYC*, *Ki-67* or *EGFR1* in comparison to fresh NK cells derived from healthy donors. NK-92 cells incubated with GC reduced the expression of positive regulators of cell proliferation, transcription factor *c-MYC* (P = 0.001), proliferation antigen *Ki-67* (P = 0.0032) and *EGFR1* (P = 0.0063) (Fig. 2B). Fresh NK cells incubated with GN4C showed decreased mRNA level of *c-MYC* (P = 0.05) (Fig. 2A) but did not express *EGFR1* or *Ki-67* genes (Fig. 2C). The decrease of *c-MYC* in fresh NK cells was less significant than in the NK-92 cell line. Together, these results demonstrate that GN4C down-modulated the expression of glycosyltransferases in the NK-92 cell line as well as in fresh NK cells and decreased the expression of cell growth regulators *EGFR1*, *Ki-67* and *c-MYC* primarily in the tumor NK-92 cell line.

The proliferation rate in both fresh NK and NK-92 cells after adding GN4C was checked by ^{3}H-thymidine incorporation assay. We used recombinant human IL-2 as a positive control of fresh NK cells proliferation. The incubation of fresh NK cells with GN4C exhibited equal increase of proliferation rate as cells incubated with IL-2 but the effect of increased proliferation lasted longer in GN4C-treated cells (Fig. 2D). The NK-92 cell line exhibited comparable rate of proliferation in untreated and GN4C-treated cells for 24 h and then GN4C slightly inhibited further proliferation of the cell line. NK-92 cells incubated in the presence of IL-2 displayed increase in proliferation (Fig. 2E).

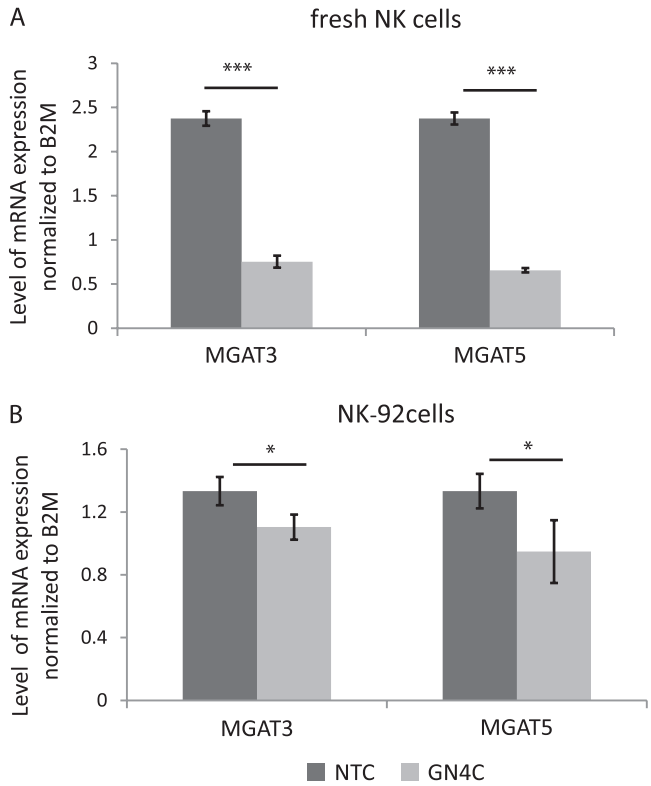

Fig. 1. Expression of glycosyltransferases *MGAT3* and *MGAT5* in fresh NK cells (A) and the NK-92 cell line (B) after incubation with GN4C. NK cells were sorted from PBMC of healthy donors and represent CD3−CD56+ population. Level of specific mRNA was detected by real-time RT-PCR and normalized to the expression of control gene *B2M*. Quantification of gene expression was performed by Bio-Rad iQ5 2.0 software and results are presented as an average ±SD of triplicates. NTC indicates non-treated control cells and GN4C indicates cells treated with GN4C.

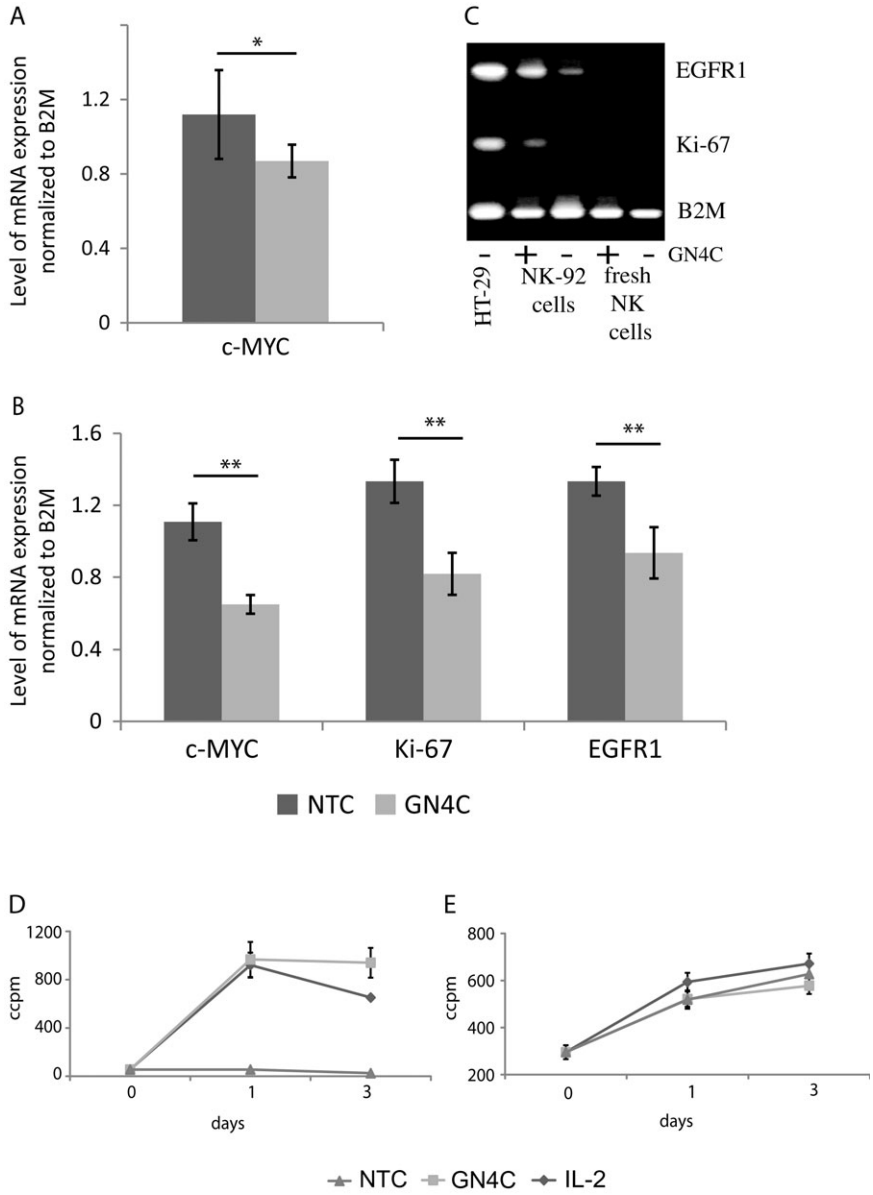

Fig. 2. Expression of cell growth regulators *c-MYC*, *EGFR1* and proliferation antigen *Ki-67* in fresh NK cells (A and C) and the NK-92 cell line (B and C) after incubation with GN4C. Level of specific mRNA was detected by real-time RT-PCR and normalized to the expression of control gene *B2M*. Quantification of gene expression was performed by Bio-Rad iQ5 2.0 software and results are presented as an average ±SD of triplicates. The HT-29 cell line (C) served as control of gene expression. Presence of GN4C is indicated by plus and absence by minus under the representative gel electrophoresis (C). Proliferation of fresh NK cells (D) and the NK-92 cell line (E) after incubation with GN4C measured by incorporation of ^{3}H-thymidine for 3 days. NTC indicates non-treated control cells, GN4C indicates cells treated with 10 nM GN4C and IL-2 indicates cells treated with 100 U of recombinant IL-2.

NK-92 and NK cells exhibited up-regulation of NKG2D

NKG2D and *NKR-P1A* genes encode activation and inhibitory NK cell receptors, respectively, and are involved in a functional regulation of NK cells. Glyco-gene array did not determined mRNA changes in these genes as significant; nevertheless, we involved them in our analysis by real-time RT–PCR that operates with higher sensitivity than high-throughput arrays. GN4C notably increased mRNA expression of C-type-lectin-like receptor *NKG2D* in the NK-92 cell line ($P = 0.0069$) as well as in fresh NK cells ($P = 0.009$). In fresh NK cells, GN4C repressed *NKR-P1A* expression ($P = 0.0075$) (Fig. 3). NK-92 cells did not express lectin receptor *NKR-P1A* either in control or in GN4C-treated cells.

GN4C increased cytotoxic activity of NK cells and susceptibility of tumor cells to NK-mediated cytolysis

Cell-mediated cytotoxicity is a primary effector function of NK cells. We previously found (11) that PBMC incubated with GN4C increased cell-mediated cytotoxicity against K562 cells. To determine if NK cell population was responsible for that cell-mediated cytotoxicity, we investigated effect of GN4C on killing potential of freshly purified NK cells and

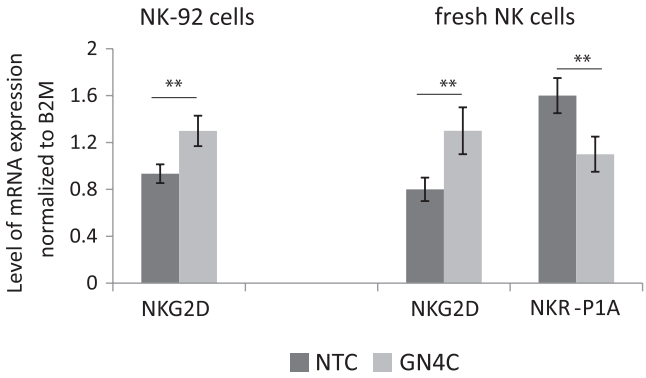

Fig. 3. Expression of NK cell receptors *NKG2D* and *NKR-P1A* in the NK-92 cell line and fresh NK cells after incubation with GN4C. NK-92 cells do not express *NKR-P1A* mRNA and thus no values are included. Level of specific mRNA was detected by real-time RT–PCR and normalized to the expression of control gene *B2M*. Quantification of gene expression was performed by Bio-Rad iQ5 2.0 software and results are presented as an average ±SD of triplicates. NTC indicates non-treated control cells and GN4C indicates cells treated with GN4C.

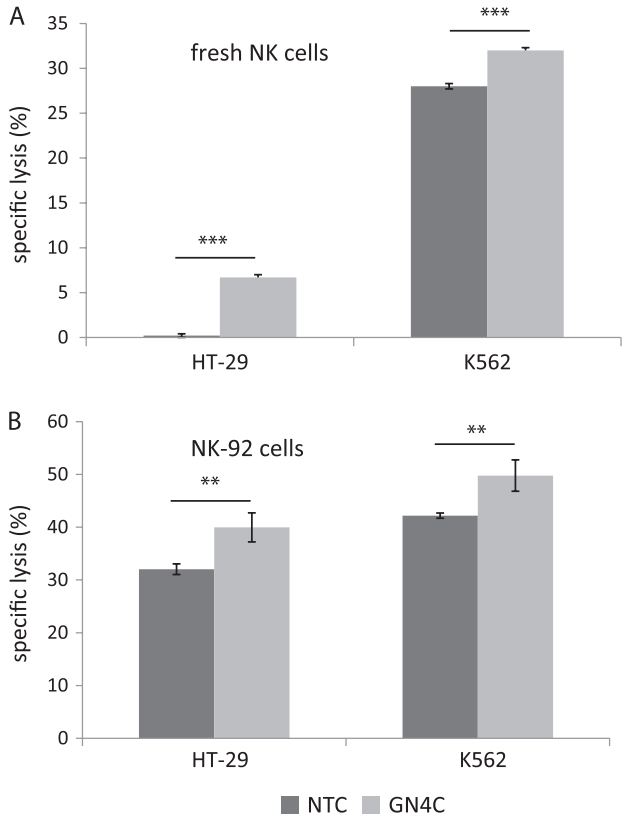

Fig. 4. Effect of GN4C on cell-mediated cytotoxicity of fresh NK cells and the NK-92 cell line. Target cells HT-29 and K562 were subjected to cytolysis by fresh NK cells (A) and NK-92 cells (B) incubated with GN4C. The effector to target (E:T) cell ratios were 10:1 and 3.5:1 for fresh NK and NK-92 cells, respectively. Non-treated cells (NTC) were used to determine basic cytolytic activity of fresh NK cells or NK-92 cells in our experimental system. Percentage of specific lyses was determined by the ^{51}Cr-release assay. Results are presented as average ±SD of triplicates.

the NK-92 cell line against sensitive K562 and resistant HT-29 tumor cells. Fresh NK cells incubated with GN4C displayed increased cytolytic activity against K562 (P = 0.0001) as well as against resistant HT-29 (P = 0.0001) target cells (Fig. 4A). We found that GN4C augmented the cytotoxicity of the NK-92 cell line against K562 (P = 0.005) and HT-29 (P = 0.0045) cells too (Fig. 4B). These results showed that GN4C promoted NK-92 and NK cell-mediated lysis against tumors of both myeloid and adenocarcinoma origin.

Further, we investigated the effect of GN4C on tumor cells, particularly their susceptibility to NK cell-mediated cytotoxicity. We used fresh NK cells as effectors against K562 and HT-29 tumor cells pre-incubated with GN4C. Both GN4C-treated tumor cell lines were more susceptible to fresh NK cell-mediated cytolysis (P = 0.0001 and 0.0383) (Fig. 5A). The same experimental design we applied to NK-92 cells as effectors against HT-29 and K562 target cells incubated with GN4C. In the case of the HT-29 cell line incubated with GN4C, we detected significant increase in cytotoxicity (P = 0.0001), nevertheless in K562 cells, the effect of GN4C was opposite (p = 0.0001) (Fig. 5B).

Pre-treatment with wortmannin effectively suppressed GN4C-mediated increase of cell-mediated cytotoxicity in both NK-92 cells (P = 0.0001 and 0.005) and fresh NK cells (P = 0.03 and 0.0005) (Fig. 8B and C). Wortmannin itself significantly reduced the basal cytotoxicity of fresh NK cells against K562 target cells (P = 0.024) and cytotoxicity of NK-92 cells against HT-29 cells (P = 0.0021). In summary, GN4C increased both the cytotoxic activity of NK cells and sensitivity of tumor cells to cytolysis. However, the cytotoxic activity of NK-92 lymphoma against GN4C-pre-treated tumor cells was not straightforward.

NK-92 cells and fresh NK cells synthesized IL-2 in response to GN4C

Besides the cytotoxicity against tumor target cells, activated NK cells also produce cytokines TNF-α and IFN-γ. To evaluate the effect of GC on cytokine synthesis by the NK-92 cell line and fresh NK cells, we measured secreted and intracellular protein levels of cytokines in control and GN4C-treated cells.

We determined the level of secreted cytokines in supernatant of the NK-92 cell line and primary culture of fresh NK cells in response to GN4C using BD™ Cytometric Bead Array. After GN4C incubation, NK-92 cells secreted significantly more TNF-α (P = 0.05), IFN-γ (P = 0.0004) and IL-2 (P = 0.0097) that are involved in T_h1 immune response. Changes in secretion of T_h2 type cytokines (IL-4 and IL-10) and IL-6 were not significant (P = 0.2, 0.31 and 0.4, respectively) (Fig. 6C). Fresh NK cells secreted significantly more IL-2 (P = 0.0001) after GN4C stimulation than untreated cells. The level of other tested cytokines (TNF-α, IFN-γ, IL-4, IL-6 and IL-10) remained unchanged (P = 0.09, 0.19, 0.8, 0.3 and 0.25, respectively) (Fig. 6D).

Since we found elevated secretion of cytokines by NK-92 and fresh NK cells, we checked their intracellular synthesis. In the NK-92 cell line, GN4C increased protein expression of TNF-α, IFN-γ and IL-2 (Fig. 6A). We found apparently increased mRNA levels for *TNF-α* and *IFN-γ* cytokines after

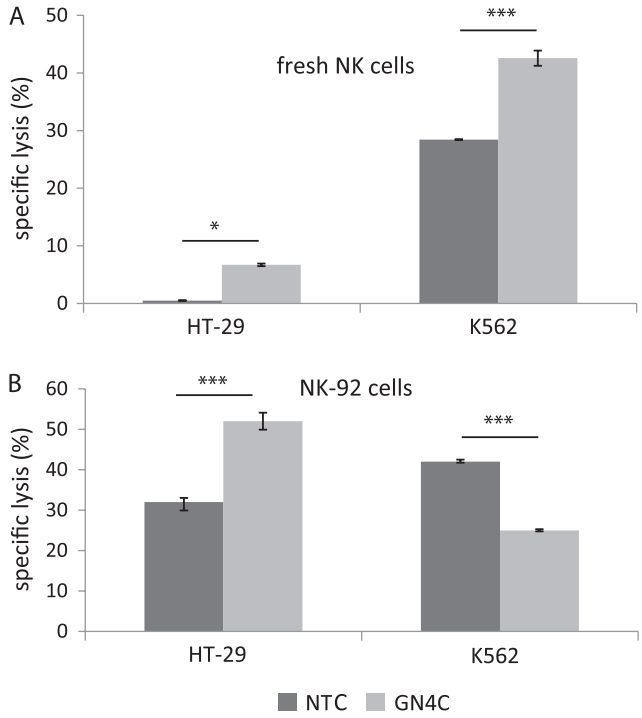

Fig. 5. Effect of GN4C on susceptibility of tumor cell lines HT-29 and K562 to cytolysis mediated by fresh NK cells (A) and NK-92 cells (B). K562 and HT-29 cells incubated with GN4C were used as targets for fresh NK cells and NK-92 cells. The effector to target (E:T) cell ratios were 10:1 and 3.5:1 for fresh NK and NK-92 cells, respectively. Non-treated tumor cells (NTC) were used as controls of NK-mediated cytotoxicity without the effect of GN4C. Percentage of specific lyses was determined by the ^{51}Cr-release assay. Results are presented as average ±SD of triplicates.

GN4C treatment as well (Fig. 6E). On the other hand, in fresh NK cells, GN4C increased intracellular level of IL-2 but not TNF-α and IFN-γ (Fig. 6B). *TNF-α* or *IFN-γ* gene expression did not change either (Fig. 6F).

To evaluate the participation of other cell types, missing in sorted NK cells, for cytokine production, we checked the intracellular TNF-α and IFN-γ synthesis in all major cell populations (CD3−CD56+; CD3+CD56+; CD3+CD56−CD4−CD8+, CD3+CD56−CD4+CD8−; monocytes) within PBMC by means of polychromatic FACS analysis. We did not detect significant changes in cytokine production in any of tested populations. TPA, used as a positive control, significantly elevated production of both cytokines in lymphocytes CD3−CD56+high, CD3+CD56+, CD3+CD56−CD4−CD8+ and monocytes (data not shown).

Signaling in NK-92 cells triggered phosphorylation of PI3K/ERK pathway

To trace signaling pathways triggered by GN4C in NK-92 cells, we examined differences in tyrosine phosphorylation with the TranSignal™ Phosphotyrosine Profiling Array. The output data set contained protein kinases with significantly up- or down-regulated tyrosine phosphorylation after GN4C treatment in comparison to non-treated control NK-92 cells. Among others, GN4C increased tyrosine phosphorylation (2.2-fold) of p85 domain 1 (subunit of PI3K) and (1.5-fold) of PLC-γ domain 1, when compared with the level of phosphorylation in control cells (Fig. 7A). Phosphorylation status of those kinases was confirmed by western blot analysis. In both, fresh NK and NK-92 cells, GN4C treatment resulted in elevated level of phosphorylated PI3K but not PLC-γ (Fig. 7B). We did not confirm the increase of PLC-γ (domain 1) mediated by GN4C as detected by TranSignal™ Phosphotyrosine Profiling Array. The difference in PLC-γ phosphorylation was higher but it still did not meet generally used criteria of 2-fold change so it might be due to technique fluctuation. In this case, western blot is more accurate since it has been optimized for detection of specific anti-phospho-PLC-γ antibody not the whole set of SH2 domains as spotted on the array.

Phosphorylation analysis was replenished with the detection of JNK1/2, ERK1/2 kinases and member of NF-κB family REL-A, which were not included on the TranSignal™ Phosphotyrosine Profiling Array. ERK1/2 and JNK1/2 molecules are involved in mediating signals resulting in cytotoxicity or cytokine production. In both, fresh NK and NK-92 cells, GN4C elevated level of phosphorylated ERK1/2 but not JNK1/2 kinases (Fig. 7B). Phosphorylation of NF-κB significantly decreased in NK-92 cells treated with GN4C but not in fresh NK cells (Fig. 7B). In conclusion, GN4C modulated signaling pathways in fresh NK and NK-92 cells through protein phosphorylation of important PI3K/ERK1/2 pathway. To support this statement, we performed experiments with PI3K inhibitor wortmannin, which reduced the amount of phosphorylated PI3K and downstream ERK1/2 (Fig. 8A) and abolished GN4C-mediated cytotoxicity in both NK-92 cells and fresh NK cells (Fig. 8B and C).

Discussion

Our previous results using PAMAM-based (polyamidoamine) GC with GlcNAc moieties for therapeutical application to tumor-bearing animals (colorectal carcinoma in rats and melanoma in mice) showed a decreased tumor growth and prolonged survival time of treated animals accompanied by enhancement of immune response (cytokine production, cytotoxicity and infiltration of tumor by activated lymphocytes) (20, 21). The introduction of calix[4]arenes in GCs as a novel scaffold bearing sugar units enabled better three-dimensional structural control than PAMAM-based GC (11) and displayed superior binding affinity than the previously tested PAMAM-GlcNAc. In animals, the results indicate structural preferences of the NK cell receptors NKR-P1 and CD69 for the GlcNAc-containing dendrimeric structures with aromatic spacers. The immunomodulatory activity results with the GlcNAc tetramers on calix[4]arene scaffold exhibit stimulation of natural cytotoxicity of human PBMC (11).

Since the GCs-like GN4C showed binding affinity especially to the NK cell receptors, we further focused on the effect of GN4C on NK cells and the NK model cell line NK-92. Glycosylation is an important protein modification changing cell appearance and function, including NK cell recognition. However, alterations of NK-92 and NK cells involving glycosylation machinery have been described only scarcely.

The present study makes several key observations regarding modulations of the NK-92 cell line and fresh NK cells by

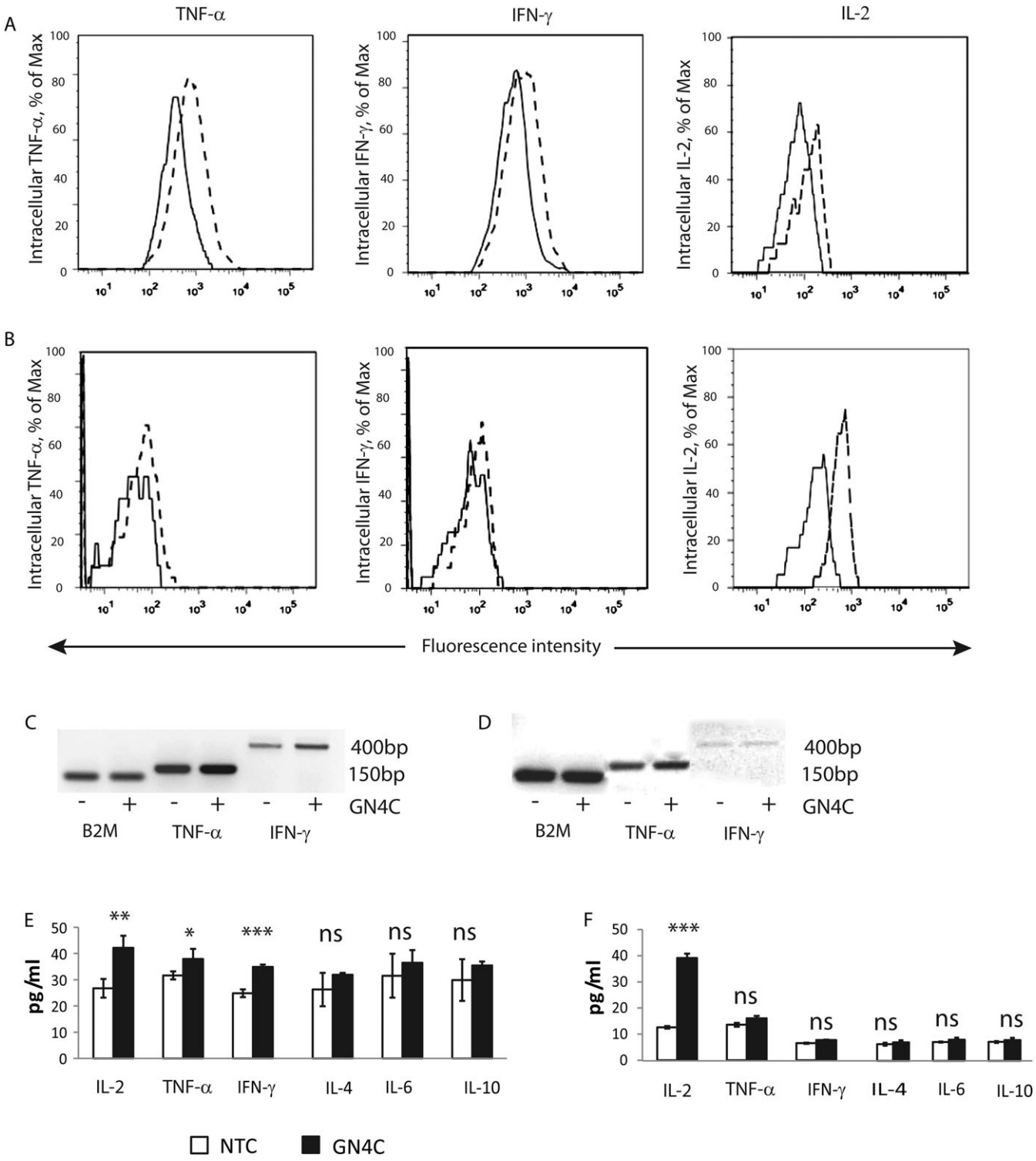

Fig. 6. Cytokine production in the NK-92 cell line (A, C and E) and sorted fresh NK cells (B, D and F) revealed with flow cytometry and RT–PCR. Intracellular production of cytokines TNF-α, IFN-γ and IL-2 in NK-92 cells (A) and fresh NK cells (B) examined by flow cytometry. Non-specific fluorescence was controlled by appropriate isotype antibodies. Expressions of cytokines are indicated by solid and dashed lines in non-treated control and in GN4C-treated cells, respectively. Data are representative of three independent experiments. Production of secreted T_h1 and T_h2 cytokines was measured by BD^{TM} Cytometric Bead Array Human $T_h1/2$ in the NK-92 cell line (C) and fresh NK cells (D). Open columns indicate untreated cells (NTC) and closed columns indicate GN4C-treated cells (GN4C). The amount of cytokines in pg ml− ±SD was determined from calibration curve of standards; Ns, not significant. Production of cytokines in NK-92 cells (E) and fresh NK cells (F) was evaluated on the level of mRNA by RT–PCR as well. Gene for *B2M* was used as a control of template amount (PCR product 150 bp). Particular cytokines are marked, as *TNF-α* and *IFN-γ* and PCR products were 166 and 396 bp long, respectively. Each lane is indicated with a plus where GN4C was added and minus where cells remained untreated. Representative figure of three independent experiments is shown.

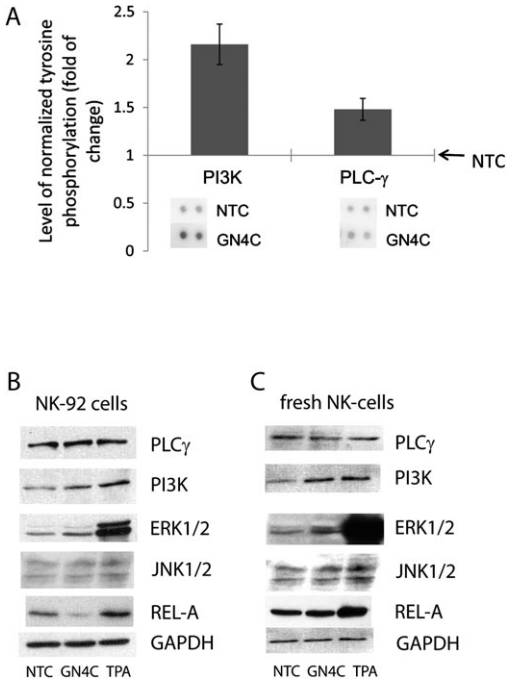

Fig. 7. Changes in tyrosine phosphorylation of PI3K and PLC-γ triggered by GN4C in the NK-92 cell line (A) as determined by a Phosphotyrosine Profiling Array, where spotted SH2 domains specifically bind phosphorylated target kinases. Each SH2 domain was tested in duplicates. Level of protein phosphorylation in control cells (NTC) is set at 1 and indicated by an arrow. Results are presented as an average ±SD of duplicates. Effect of GN4C on tyrosine phosphorylation of individual proteins PLC-γ, PI3K, ERK1/2, JNK1/2 and REL-A (NF-κB) was further analyzed by western blot in NK-92 and fresh NK cells (B). Immunodetection was performed with appropriate antibodies as outlined in Methods. GAPDH antibody was used as a protein load control. Representative image of three independent experiments is shown. TPA served as positive control for MAPK activation. NTC indicates non-treated control cells and GN4C indicates cells incubated with GN4C.

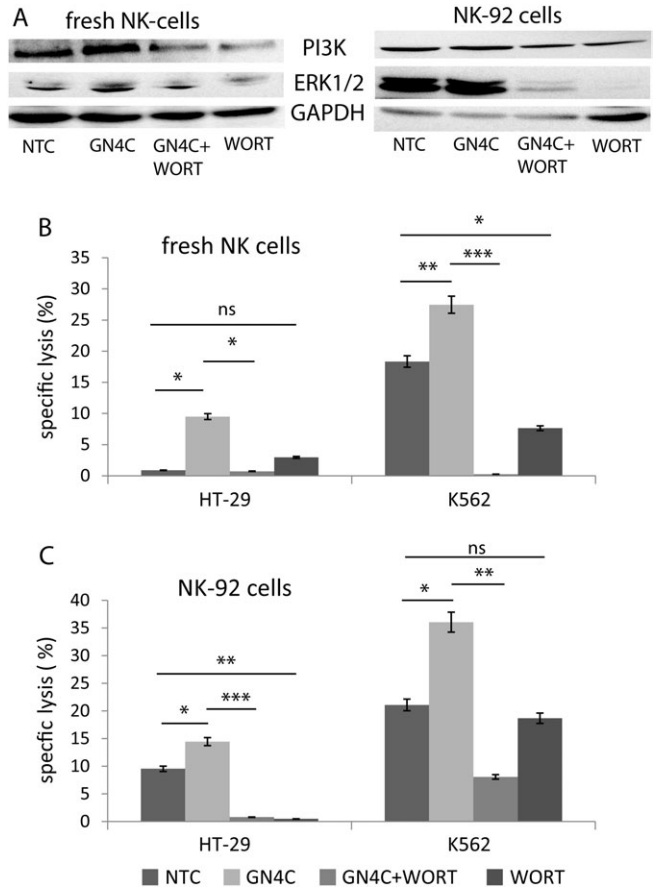

Fig. 8. Immunodetection of PI3K and ERK1/2 in NK-92 cells and fresh NK cells pre-treated with PI3K inhibitor wortmannin (A). GAPDH antibody was used as a protein load control. Representative image of three independent immunoblots is shown. Target cells HT-29 and K562 were subjected to cytolysis by fresh NK cells (B) and NK-92 cells (C) incubated with GN4C, wortmannin or combination of both. The effector to target (E:T) cell ratios were 10:1 and 3.5:1 for fresh NK and NK-92 cells, respectively. Percentage of specific lyses was determined by the ^{51}Cr-release assay. Results are presented as average ±SD of triplicates. NTC indicates non-treated control cells, GN4C indicates cells incubated with GN4C, GN4C + WORT indicates cells pre-treated with wortmannin and subsequently treated with GN4C and WORT indicates cells incubated with wortmannin alone.

GC GN4C. First, we demonstrated that GN4C interfered with the glycosylation processes of NK-92 and fresh NK cells. This compound suppressed the expression of glycosyltransferases *MGAT3* and *MGAT5*, the key enzymes involved in terminal glycan elongation. Second, we found functional activation of fresh NK cells and the NK-92 cell line by GN4C, which correlated with increased mRNA expression of activation receptor *NKG2D*. Third, GN4C increased susceptibility of tumor cells to NK cell-mediated cytolysis in fresh NK cells. In parallel, GN4C lessened the expression of cellular proto-oncogenes *c-MYC*, JUND, EGFR1, *TGF-β1*, *VEGF-B* and proliferation-associated marker *Ki-67* in tumor NK-92 cells. Overall, the present study provides evidence that GN4C is a potent modulator of fresh NK cells as well as the NK-92 cell line.

Glycosyltransferases MGAT3 and MGAT5 are competitors driving terminal glycan elongation, and their activity is controlled by gene expression (22). Therefore, down-regulation of glycosyltransferases by GN4C may promote recognition capability of fresh NK cells and the NK-92 cell line and contribute to enhanced functional activity. The effect of GN4C on NK cell-mediated cytotoxicity and cytokine synthesis was lower in fresh NK cells than in the NK-92 cell line. This difference might be a result of lacking inhibitory receptors in NK-92 cells and thus higher effector function of the cell line. Inhibitory receptor *CD161* (*NKR-P1A*) exhibited reduction of its expression after GN4C treatment (Fig. 3), which supported the activation of NK cells. In contrast to the NK-92 cell line, fresh NK cells exhibit also whole family of KIR inhibitory receptors that may reduce GN4C-mediated activation signal (23, 24).

Human NK cells require IL-2 to activate their antitumor cytotoxic response. Saito *et al.* (25) described expression of endogenous *IL-2* mRNA in NK cells. IL-2 secreted by NK cells after GN4C incubation might serve as the autocrine factor for proliferation and cytolysis enhancement.

Cancer cells surface possesses many glycosylated structures and we supposed that tumor cells could bind the GC

as well. GN4C binding by tumor cells and subsequent modulation of *MGAT* glycosyltransferases expression may change tumor cells recognition pattern and make them more attractive for immune cells. In functional cytotoxicity assay performed with GN4C-pre-treated tumor targets, we found higher cytolysis than in the experiment with GN4C pre-incubated effector cells. NK cells kill preferentially hematopoietic cells, whereas many tumors derived from other tissues are resistant to NK cells (26). Thus, GN4C increased susceptibility of the HT-29 adenocarcinoma cell line to fresh NK cell-mediated killing is of remarkable importance. Using NK-92 cells as effectors against GN4C-pre-treated HT-29 adenocarcinoma cells, we obtained increase of HT-29 cytolysis. When we applied this approach to GN4C-pre-incubated K562 leukemia cells, we detected decrease of NK-92-mediated cytolysis. We suppose that K562 may express different glycan pattern that may bind GN4C and trigger intracellular signal or mask ligands for NK-92 recognition and thus protect tumor cells from NK-92 cytolysis.

NK cell activity is triggered by NK cell receptors and further mediated through kinase phosphorylation including PI3K/ERK or PLC-γ/JNK molecules (7, 9). GN4C binds to rat NKR-P1A, which is NK cell rodent activation receptor; nevertheless, in human, NKR-P1A serves as inhibitory receptor (5). Since GN4C resulted in increased NK cell effector functions, another receptor triggering activation signal (NKG2D) has to be involved.

GN4C triggered signal through PI3K/ERK pathway resulting in NK cell cytotoxic activity. This finding is in an agreement with the description of pathway leading to activation of NK cell functions described earlier (9). The decrease of GN4C-induced cytotoxicity in NK-92 cells and fresh NK cells by PI3K inhibitor supported the involvement of PI3K/ERK pathway in GN4C-triggered enhancement of cytotoxicity. We hypothesized that NKG2D was the activation receptor triggered by GN4C because of its increased expression. JNK kinase, which is downstream of PLC-γ, is required for NKG2D-triggered NK cell cytotoxicity (7). In our experiments, GN4C did not enhance JNK phosphorylation, which excludes NKG2D to be GN4C-binding activation receptor. Therefore, the discovery of a GN4C-responsive activation receptor leading to cell cytotoxicity without JNK involvement is a challenge for future experiments. Less abundant changes in protein phosphorylation of fresh NK cells reflect the presence of NK cell inhibitory receptor NKR-P1A or KIRs that may bind GN4C simultaneously and thus reduce the activation signal strength as mentioned above.

Since NK-92 cells are originally lymphoma cells, GN4C-mediated down-regulations of glycosyltranferases *MGAT3*, *MGAT5* and especially proto-oncogenes *c-MYC*, REL-A, *EGFR1*, *VEGF-B* and *TGF-β1* were tremendously important. The expression of growth factors often alters in human cancer, and their over-expression correlates with tumor progression. Recently, a new role for EGFR1 in cancer has appeared; in tumors of epithelial origin, EGFR1 associates and stabilizes sodium/glucose cotransporter SGLT1. Down-regulation of *EGFR1* leads then to a loss of *SGLT1* expression and low intracellular glucose levels (13). Given that GN4C interferes with cell glycosylation, we examined the glucose uptake transporter *SGLT1/EGFR1* in fresh NK and NK-92 cells. We found decreased expression of important cellular proto-oncogene *EGFR1* in NK-92 cells but no detectable *SGLT1* gene. So a different transporter than SGLT1/EGFR1 mediated the glucose uptake in fresh NK and NK-92 cells.

Moreover, we found that GN4C GC inhibited expressions of *TGF-β1* and *VEGF-B*, positive regulators of neoangiogenesis, the fundamental requirement for cancer progression (27, 28). Calix[4]arene compounds show inhibition of angiogenetic factors *in vitro* as well as *in vivo* in mouse models (29). Therefore, our results confirmed and extended previous findings indicating that calix[4]arene-based GCs acted as regulators of neoangiogenesis via inhibition of TGF-β1 and VEGF-B (29).

In our opinion, the down-regulation of proliferation markers by GN4C is independent to increased cytotoxic function of the NK-92 cell line or fresh NK cells. The tested GC consists of two different chemical structures that can trigger different signaling pathways. We suppose that GlcNAc part is responsible for higher cytotoxicity and calix[4]arene core is responsible for the inhibition of expression of *Ki-67*, *EGFR*, *c-MYC* and other tumor markers (*VEGF-B* and *TGF-β1*) as we observed in further experiments with different tumor cell lines (V. Benson, V. Grobarova, J. Richter and A. Fiserova, unpublished data).

In conclusion, the modulation of glycosyltransferases *MGAT3* and *MGAT5* by chemically defined synthetic GC GN4C correlated with the improvement of NK cell effector functions and the augmentation of tumor cell sensitivity to NK cell-mediated cytotoxicity. These results demonstrate the great potential of the GN4C compound as a NK cell-mediated antitumor response modulator.

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3.3 Collagen-induced arthritis: severity and immune response attenuation using multivalent GlcNAc

3.3.1 Overview

Rheumatoid arthritis (RA) is a systemic autoimmune disease where conventional therapeutic modalities bring a range of adverse effect, so there is an urge to seek novel disease modulators [199, 200]. N-Acetyl-glucosamine, currently used as a supplement for osteoarthritis treatment, was shown to possess no side effects even at high doses [201] and researchers have recently reported its beneficial effects in animal arthritic models [200, 202]. As multivalence plays an important role in glycan interaction with its corresponding receptors, we used multivalent glycodendrimers bearing 4 or 8 GlcNAc moieties to treat collagen-induced arthritis in DBA/1 mice. These conjugates were previously reported to affect NK and B cell function [194, 195, 203] and down-regulate MGAT5 gene expression in synovial cells from RA patients (Publication 1 [204]), fresh human NK cells and NK92 cell line (Publication 2 [205]). The role of NK cells in RA and CIA is not fully understood, with evidence that they can both contribute to or protect against inflammation [206]. NK cells were reported to support the ongoing inflammation in the joints particularly by cytokine production and interaction with other pro-inflammatory cells [155, 207]. On the contrary, papers suggesting a protective role were also published describing suppression of pro-inflammatory Th17 cells by means of NK-cell derived IFN-γ [160].

3.3.2 Aims and methods

The aim of our study was to evaluate the effect of GN8P and GN4C glycoconjugates on CIA-bearing mice and their potential use as disease-modulating agents. We induced CIA in DBA/1 mice kept under SPF conditions and treated them with the conjugates administered by intraperitoneal injection. Clinical scoring and immunohistochemistry were used to evaluate the joint condition and overall disease progression. The immunological parameters were followed by flow cytometry, cytometric bead array (cytokine production), ELISA (disease-specific antibodies), RealTime RT-PCR (cytokine expression) and cytotoxicity assay (NK cell function).

3.3.3 Results and discussion

In this study, we showed a significant amelioration of rheumatic symptoms in CIA mice after the glycoconjugate (GC) treatment corresponding with similar effects of pure GlcNAc [200]. Our treatment prevented the inflammatory response in situ by moderation of the immune infiltration and reduction of both monocyte- and B cell-derived antigen-presenting cells (APC), T cells and NKG2D positive NK cells in the synovial fluid. The CD86-positive B cell-derived APCs are crucial for the activation of autoreactive T cells in RA [208], so their reduction is highly favourable. The reduction of activated NK cells corresponds with the beneficial effect of antibody-mediated

NKG2D blockage demonstrated previously [127, 209]. The difference between our two conjugates in terms of NKG2D reduction corresponded with the difference in the clinical effect where GN4C was more potent. As for NK cell effector function, we confirmed the previously reported impairment that appears not only in CIA [160] but also in human RA [152]. We also found a corresponding reduction of NK cell relative distribution in the spleen.

Evaluating the effects of the treatment on humoral response, we found slightly reduced titre of specific anti-CII IgG2a antibody after the treatment. IgG2a is a Th1-driven isotype induced by IFN-γ, so we also tested this cytokine along with TNF-α which is a major cytokine driving arthritic inflammation [210, 211]. Our GC treatment successfully prevented the rise of IFN-γ production corresponding with the above mentioned reduction of specific IgG2a. TNF-α production was also significantly attenuated, which, along with the reduction of inflammatory infiltration, resembles a favourable clinical effect of a direct blockage of TNF-α [212]. Anti-TNF therapy used in RA patients also reduced CD86 expression on B cells that is also in accord with our results [213].

Concerning the role of anti-inflammatory cytokines that did not show elevated levels in blood, we tested the expression of IL-4 in splenocytes. IL-4 is a crucial anti-inflammatory mediator of CIA, correlating with tolerance induction [214]. Our GC treatment caused a significant increase of its expression that correlated with the proposed function as an important suppressor of CIA development.

Taken together, the GC treatment is capable of effective reduction of the incidence and postponement of the CIA onset as well as moderation of its severity. This is caused particularly by the prevention of inflammatory infiltration, inhibition of antigen presentation by both B cells and professional APCs and suppression of proinflammatory cytokine production. We suggest that inhibition of NKG2D-positive NK cells plays an important role here as well, leading to the moderation of autoimmune processes. We assume a complex effect of GlcNAc glycoconjugate treatment orchestrating interaction of both innate and adaptive immune responses and leading to attenuation of the disease clinical symptoms. Therefore, we assume that GlcNAc, and particularly its multivalent forms, could serve as a promising agent in the supportive treatment of rheumatoid arthritis early stages.

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Collagen-induced arthritis: severity and immune response attenuation using multivalent N-acetyl glucosamine

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Summary

Rheumatoid arthritis is an autoimmunity leading to considerable impairment of quality of life. N-acetyl glucosamine (GlcNAc) has been described previously as a potent modulator of experimental arthritis in animal models and is used for osteoarthritis treatment in humans, praised for its lack of adverse effects. In this study we present a comprehensive immunological analysis of multivalent GlcNAc-terminated glycoconjugate (GC) application in the treatment of collagen-induced arthritis (CIA) and its clinical outcome. We used immunohistochemistry and FACS to describe conditions on the inflammation site. Systemic and clinical effects were evaluated by FACS, cytotoxicity assay, ELISA, cytometric bead array (CBA), RT–PCR and clinical scoring. We found reduced inflammatory infiltration, NKG2D expression on NK and suppression of T, B and antigen-presenting cells (APC) in the synovia. On the systemic level, GCs prevented the activation of monocyte- and B cell-derived APCs, the rise of TNF-α and IFN-γ levels, and subsequent type II collagen (CII)-specific IgG2a formation. Moreover, we detected an increase of anti-inflammatory IL-4 mRNA in the spleen. Similar to the synovia, the GCs caused a significant reduction of NKG2D-expressing NK cells in the spleen without influencing their lytic function. GCs effectively postponed the onset of arthritic symptoms, reduced their severity and in 18% (GN8P) and 31% (GN4C) of the cases completely prevented their appearance. Our data prove that GlcNAc glycoconjugates prevent the inflammatory response, involving proinflammatory cytokine rise, APC activation and NKG2D expression, leading to the attenuation of clinical symptoms. These results support the glycobiological approach to the treatment of collagen-induced arthritis/rheumatoid arthritis (CIA/RA) as a way of bringing new prospects for more effective therapeutic interventions.

Keywords: CIA, clinical scoring, cytokines, GlcNAc glycoconjugates, humoral response

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation resulting in subsequent cartilage and bone destruction [1]. Conventional therapeutic modalities used for the treatment of RA have significant side effects [2,3], so there is a need to seek novel disease modulators. As the autoreactivity of immune cells is elicited in part by the dysregulated glycosylation, research during the past two decades has focused upon studying the molecular mechanisms underlying these changes and their role in autoimmune rheumatic diseases [4,5].

N-acetyl-glucosamine (GlcNAc) is widely used as a supplement for treating osteoarthritis (its beneficial effects were proved in a placebo-controlled clinical trial [6]) and

was reported to possess no adverse effects, even at high doses [7]. For RA, trials were performed with pure glucosamine only [8]. Suppressive effects of GlcNAc on experimental adjuvant arthritis in rats were reported by Hua *et al.* [9], and recently, Azuma *et al.* have described similar results in a laminarin-induced model of human RA in SKG/Jcl mice [3]. All the studies mentioned were performed with GlcNAc monosaccharide known to have low binding affinity to corresponding receptors. Multivalence thus plays an important functional role in carbohydrate–protein interactions [10]. Glycodendrimers with four (GN4C) and eight (GN8P) GlcNAc moieties on calix[4]arene and polyamidoamine scaffold, respectively, showing different three-dimensional (3D) structure and rigidity, were reported previously to modulate natural killer (NK) and B cell function [11–13], down-regulate the MGAT5 (mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase) gene expression in synoviocytes of RA patients (GN8P) [14] and in fresh NK cells and the NK92 cell line (GN4C) [15]. We also found that GN8P influenced the NK cells isolated from RA-affected joints of the patients and that GlcNAc-carrying synthetic dendrimers have the ability to modulate the cytotoxic activity of NK cells [14] in a receptor repertoire-dependent manner; nevertheless, the exact mechanism remains unclear.

Collagen-induced arthritis (CIA) is an established experimental model of human RA induced by immunization of mice with bovine or chicken type II collagen (CII) emulsified in complete Freund's adjuvant (CFA) that results in rheumatic symptoms such as joint inflammation and swelling [16]. Unlike other models, such as collagen antibody-induced arthritis, that only elicits the effector functions leading to the rheumatic symptoms without the preceding immunopathology [17], the CIA model is suitable for studying the alterations of immune functions during disease development and progression.

The role of NK cells in RA and CIA is not fully understood, with evidence that they can both contribute to or protect against inflammation [18]. De Matos *et al.* showed a strong up-regulation of cytokine-producing NK cells ($CD56^{bright}$) in the synovial fluid of RA patients supporting the ongoing inflammation [19]. Corresponding results were also presented by Dalbeth and Callan, who suggested that NK cells in the inflamed synovium interact with the macrophage/monocyte population, thus amplifying the production of inflammatory cytokines [20]. Conversely, Aramaki *et al.* reported low, impaired NK activity in human RA [21]. Lo *et al.* even reported that NK cells play a protective role in the development of CIA, which is mediated by interferon (IFN)-γ production suppressing the generation of Th17 cells [22]. Moreover, NK cells play different roles during different stages of the disease [23]. We were thus interested in the function of NK cells during the development of CIA and whether it can be modulated by the *in-vivo* administration of glycoconjugates that had previously been effective in alteration of NK cell function in rheumatoid arthritis *in vitro* [14]. The aforementioned effects of simple GlcNAc prompted us to examine the multivalent GlcNAc glycoconjugate efficacy, assuming their higher binding affinity to respective ligands on rheumatoid arthritis and its animal models.

The aim of our study was to evaluate the effect of GN8P and GN4C on CIA-bearing mice and their potential use as disease-modulating agents. First, we tested the GN8P treatment in two different administration schemes and focused on local reaction in arthritic joints by immunohistochemistry and FACS analysis of the synovial fluid with particular respect to NK, B and antigen-presenting cells. GN4C, a glycoconjugate with a more rigid structure, was added during the course of the study and compared with GN8P in terms of its systemic effect on antigen-presenting cells, NK and B cells and the clinical outcome. To evaluate the effect of the treatment, we employed clinical scoring [24], cytotoxicity assays, FACS, ELISA, real-time RT–PCR and cytometric bead array (CBA).

Materials and methods

Experimental animals

Ten-week-old male and female DBA/1 mice (Taconic, Lille Skensved, Denmark) were housed under specific pathogen-free conditions (22°C, 55% relative humidity, 12-h day/night cycle) and fed on NIH#31 rodent diet (Taconic) *ad libitum*. All procedures were conducted in accordance with the European Convention for the Care and Use of Laboratory Animals, as approved by the Czech Animal Care and Use Committee.

N-acetyl-D-glucosamine-coated dendrimers

Two structurally different glycodendrimers bearing four or eight GlcNAc moieties (Fig. 1a, GlcNAc monosaccharide) were used in this study. GN8P is a glycodendrimer based on a polyamidoamine scaffold bearing eight GlcNAc molecules (Fig. 1b). GN4C carries four GlcNAc molecules on calix[4]arene scaffold and is thus more rigid in its 3D structure (Fig. 1c). Both dendrimers were synthesized as described previously and kindly provided by Professor Lindhorst (CAU, Kiel, Germany) and Professor Kren (MBU, Prague, Czech Republic). The structure and purity was confirmed by electrospray ionization mass spectrometry and nuclear magnetic resonance [13,25].

Induction of CIA

CIA was induced as described by Brand *et al.* [16]. The animals, fixed in a restrainer, were immunized by

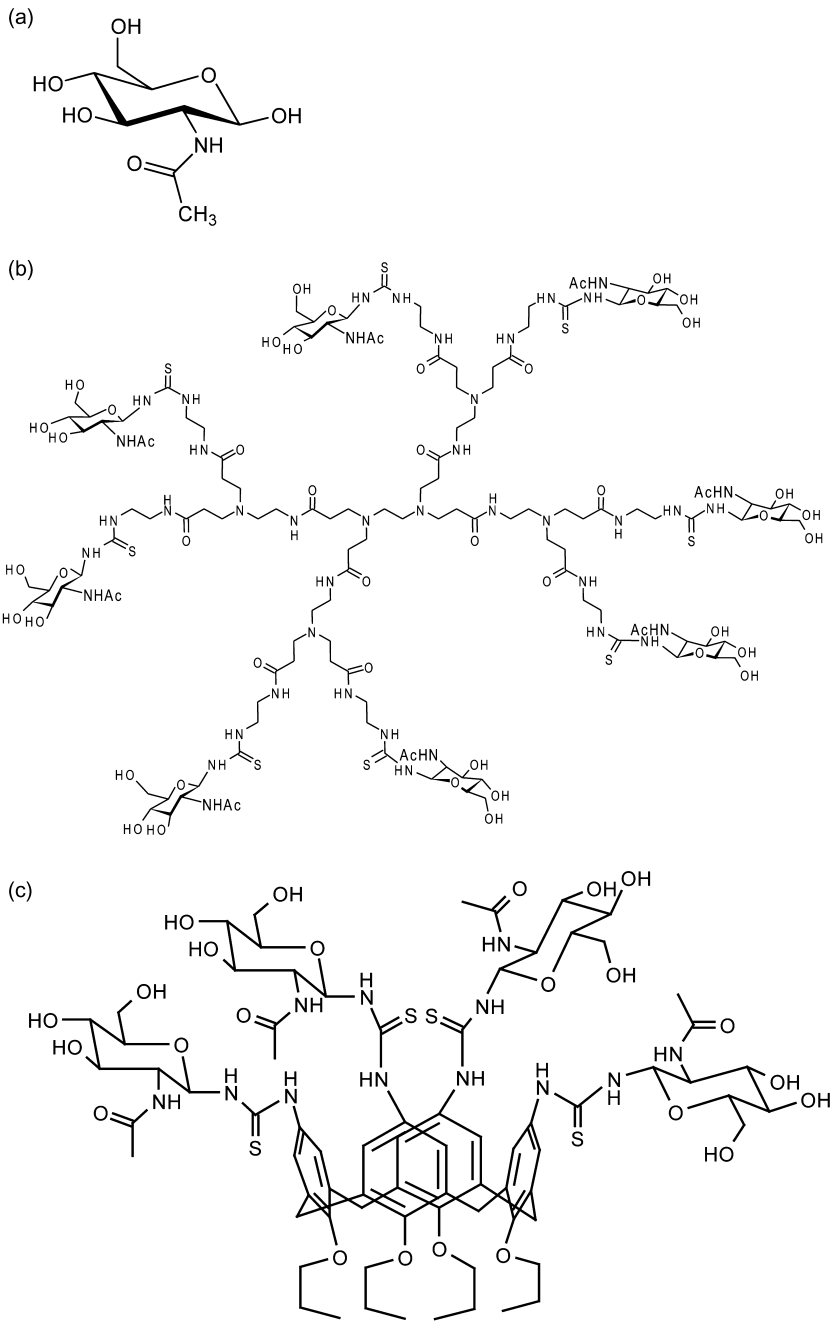

Fig. 1. Chemical structures of N-acetyl glucosamine (GlcNAc) and glycodendrimers. 2-(acetylamino)-2-deoxy-D-glucose or GlcNAc (a) is the simple monosaccharide derivative of glucose. GN8P (b) is a glycodendrimer bearing eight GlcNAc moieties on polyamidoamine (PAMAM) scaffold. GN4C (c) is a glycodendrimer bearing four GlcNAc moieties on calix[4]arene core providing a more rigid structure than PAMAM.

intradermal (i.d.) injection in the proximal third of the tail with 200 μg of bovine type II collagen (CII; Chondrex, Redmond, WA, USA) freshly dissolved in 0·1 M acetic acid and emulsified in equal volumes of complete Freund's adjuvant (CFA; Sigma-Aldrich, St Louis, MO, USA) fortified with 4 mg of *Mycobacterium tuberculosis* strain H27Ra per ml. The emulsion was prepared immediately before immunization by thorough mixing in a 0·5 ml syringe.

Glycoconjugate administration

Glycoconjugates (0·15 mg/kg) were administered intraperitoneally either in presymptomatic (days 10, 13, 16, 23 and 30) or symptomatic (days 23, 26, 29, 36 and 43) treatment schedules (Supporting information, Fig. S1). The concentration used was in accordance with the standard treatment dosage established and proved effective for immune modulation in our previous *in-vivo* studies

[11–13]. Animals in the healthy control (HC) and untreated CIA (CIA) groups were injected with equal volumes of sterile phosphate-buffered saline (PBS) at the same time-points. The study comprised experimental groups of five to 10 animals in three to five independent experiments.

Histology and immunohistochemistry

Limbs from control and CIA mice were removed at euthanasia; joint tissue was micro-dissected, embedded in Jung tissue-freezing medium (Leica, Wetzlar, Germany) and frozen immediately in liquid nitrogen. Four-μm sections were fixed on lysine precoated slides in acetone and stained. Anti-CD11b-biotin and anti-NKG2D-biotin-conjugated primary antibodies (eBioscience, San Diego, CA, USA) were used. After 2-h incubation at 4°C the sections were washed and developed using a Vectastain ABC kit and diaminobenzidine (Vector, Burlingame, CA, USA), according to the manufacturer's protocol. Control slides for background and unspecific staining were prepared using normal rat serum instead of primary antibody, following the same procedures. Haematoxylin was used for counterstaining. Diagnosis was performed at ×10 , ×20 and ×40 magnifications (Zeiss transmission light microscope) by an expert pathologist. Microphotographs were obtained at ×40 magnification.

Isolation of spleen mononuclear cells (SMCs), lymph node cells and synovial fluid cells

Spleens were squeezed through nylon mesh and separated on Ficoll-Hypaque (Sigma Chemicals/Sigma Aldrich, St Louis, MO, USA) density gradient (1·086 g/ml: optimal density for murine leucocyte isolation). SMCs were washed three times in HEPES minimum essential medium (H-MEMd) (IMG, Prague, Czech Republic), resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 0·05 mg/ml gentamycin (IMG) and 5% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), and used immediately for assays. Lymph node cells were isolated in the same manner, omitting density gradient separation. Synovial fluid leucocytes from the arthritic paws were obtained after repeated intensive lavage of joints using H-MEMd, further washed and used for FACS analysis.

Flow cytometry (FACS)

Heparinized blood samples were seeded into U-bottomed 96-well microtitre plates (Nunc, Roskilde, Denmark) and erythrocytes were lysed using 0·15 M ammonium chloride buffer (22°C, 12 min). The cells were centrifuged (400 ***g*** for 2 min) and washed three times in ice-cold PBS containing 0·02% cold-water fish-skin gelatine and 0·01% sodium azide (Sigma-Aldrich). Cell suspensions prepared from spleens, lymph nodes and synovial fluid of individual mice were resuspended in the same PBS. The following monoclonal antibodies were used according to the manufacturer's protocol: CD45R/B220-Pacific Orange, CD3-phycoerythrin (PE), Nkp46-fluorescein isothiocyanate (FITC), CD11b-AlexaFluor 700, NKG2D-biotin, CD86-allophycocyanin (APC), CD11c-FITC, IA-IE-PE (BD Biosciences, San Jose, CA, USA or eBioscience, San Diego, CA, USA). We detected biotin by streptavidin-Qdot605 (Invitrogen, Grand Island, NY, USA). Samples were measured by BD LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) in a four-laser set-up (405 nm, 488 nm, 561 nm and 633 nm) and data were evaluated and offline-compensated based on single-stain controls in FlowJo version 9 (Tree Star, Ashland, OR, USA). Morphology and doublet exclusion was based on forward-scatter (FSC) area, FSC height and side-scatter (SSC) area; we used propidium iodide (BD Biosciences) for exclusion of non-viable cells.

Cytometric bead array (CBA)

Cytokine levels were measured by mouse Th1/Th2/Th17 CBA kit (BD Biosciences). Fluorescent beads of various sizes and fluorescence intensities coated with anti-cytokine antibodies were incubated with the serum samples, and a detection antibody conjugated with a different fluorochrome was used to detect the amount of the bound analyte. Serum samples were obtained by centrifugation of collected blood, processed according to the manufacturer's protocol, and measured on a BD LSRII. Data were evaluated in FlowJo version 9 and the median of fluorescence intensity was used to determine cytokine levels. Concentrations were calculated based on a standard curve.

Real-time RT–PCR

RNA was isolated from splenocytes with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and transcribed into cDNA using a cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time RT–PCR was carried out with FastStart SYBR Green Master mix (Roche, Mannheim, Germany) in iCycler5 (Bio-Rad, Hercules, CA, USA). Interleukin (IL)-4 primer was designed in Primer3 software (forward primer: ACAGGAGAAGGGACGCCAT, reverse primer: TGAGCTCGTCTGTAGGGCTTC). Gene expression was normalized to β2-microglobulin (GeneriBio, Hradec Kralove, Czech Republic). Real-time RT–PCR data were evaluated in iQ5 softare (Bio-Rad).

ELISA for specific anti-collagen II (CII) IgG2a

Flat-bottomed, 96-well microtitre Maxisorp plates (Nunc) were coated with bovine CII (used for the induction of CIA) at 10 μg/ml in 0·1 M $NaHCO_3$ (100 μl/well), incubated overnight at 4°C and blocked with 5% BSA–PBS

(22°C, 2 h). Three dilutions (×10, ×100 and ×1000) of each sample were added in duplicate (100 μl/well). After overnight incubation (4°C) and extensive washing, anti-CII IgG2a was detected with horseradish peroxidase-conjugated goat anti-mouse IgG2a (Jackson, West Grove, PA, USA). Plates were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD, USA). Optical density was read at 450 nm by a Rainbow Thermo ELISA reader (Tecan, Salzburg, Austria). Sera from non-immunized mice (HC group) were used as controls.

Cytotoxicity assay

Cell-mediated cytotoxicity of SMC was estimated by the ^{51}Cr-release assay, as described previously [26]. YAC-1 target cell line (mouse NK-sensitive T lymphoma) was maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0·05 mM 2-mercaptoethanol antibiotics (0·05 mg/ml gentamycin, 25 mg/ml amphotericin B) (IMG) and 10% heat-inactivated fetal calf serum (Biochrom). SMCs isolated from mice (effector cells, E) were pipetted into U-bottomed 96-well microtitre plates (Nunc). Then YAC-1 target cells (T) labelled with $Na_2{}^{51}CrO_4$ were added at an effector : target (E : T) ratio of 32:1 and incubated for 18 h (37°C, humidified atmosphere, 5% CO_2) (Jouan, St Herblain, France). Cell-free supernatants were harvested (25 μl/sample) and mixed with 75 μl/sample of scintillation cocktail (SuperMix; Wallac, Turku, Finland). Radioactivity was measured using a Microbeta Trilux scintillation counter (Wallac). Effector cell lytic activity against target cells was calculated as follows: %cytotoxicity = 100 × (cpm) (specific lysis) − cpm (spontaneous lysis)]/[cpm (maximum lysis) − counts per minute (cpm (spontaneous lysis)].

Arthritis scores

Paws of individual mice were scored for clinical signs of arthritis three times a week based on standard scoring protocol: 0 = healthy/normal, 1 = erythema and swelling in digits, 2 = erythema and swelling in digits and metatarsal joints, 3 = erythema and swelling in digits, metatarsal and tarsal joints and 4 = erythema and severe swelling affecting the whole paw. Data are expressed as mean number of arthritic limbs per group, mean score per arthritic limb in the group, average symptom onset time and percentage of symptom-bearing animals [24].

Statistical analysis

The comparison of subgroups was based on various models of analysis of variance. Mouse was regarded as a random factor nested within the subgroup. A repeated-measure design was used to account for within-subject changes of score in time. Time to clinical onset was modelled by Cox's proportional hazards regression. Categorical data were described using absolute and relative frequencies and compared by Fisher's exact test. All statistical tests were treated as two-sided and evaluated at a significance level of 0·05. Results represent averages of multiple experiments and are expressed as mean ± standard deviation (s.d.). Statistical analysis was performed in Stata version 9·2 (StataCorp, College Station, TX, USA); graphs were produced by Prism 5 (GraphPad, San Diego, CA, USA) and Excel 2010 (Microsoft, Redmond, WA, USA).

Results

Our previous research concentrated on the role of NK cells from different stages of human RA and their modulation by *in-vitro*-added GN8P glycoconjugate [14]. In the study presented herein, we were interested in the local reaction in rheumatic joints after *in-vivo* administration of GN8P in the animal model of autoimmune arthritis: CIA. We tested presymptomatic and symptomatic administration schemes and found that in the symptomatic schedule, the GC treatment had no substantial effect on either the clinical outcome or the followed immune parameters. We thus concentrated on the presymptomatic treatment starting at day 10, when the inflammatory response peaks, showing promising results presented thereafter. All data were first evaluated statistically with regard to the gender of the experimental animals. After we found no significant differences between males and females (represented 1:1) within the parameters of our interest, we processed the data obtained from both genders combined.

Attenuation of histological and immunological characteristics in CIA-affected joints by GN8P

Histological samples of the joint soft tissue demonstrated intensive inflammatory cellular infiltration under CIA conditions in comparison with healthy tissue, and more moderated infiltrate after GN8P treatment (Fig. 2a–c). The immunohistochemical staining of NKG2D and CD11b for the evaluation of specific cell types showed no cells expressing NKG2D receptor in the joints of healthy mice, whereas they were scarcely present in the CIA infiltrate (Fig. 3a,b). Cells expressing CD11b were rare in the healthy joint tissue, but abundant and confluent within the inflammatory infiltrate in untreated CIA mice (Fig. 4a,b). GN8P treatment did not cause marked changes in the NKG2D$^+$ infiltration (Fig. 3c), while the CD11b-positive cells were still present in clusters but less massively than in the untreated CIA mice (Fig. 4c).

In the synovial fluid, we detected a non-significantly lower proportion of NKG2D-positive cells after the GN8P treatment; however, NKG2D expression (MFI) on NK cells (CD3$^-$NKp46$^+$) was decreased significantly (Fig. 5a,b). We found a slight reduction of CD11b$^+$ cell infiltration, while the CD11b$^+$CD11c$^+$ antigen-presenting cell subset was

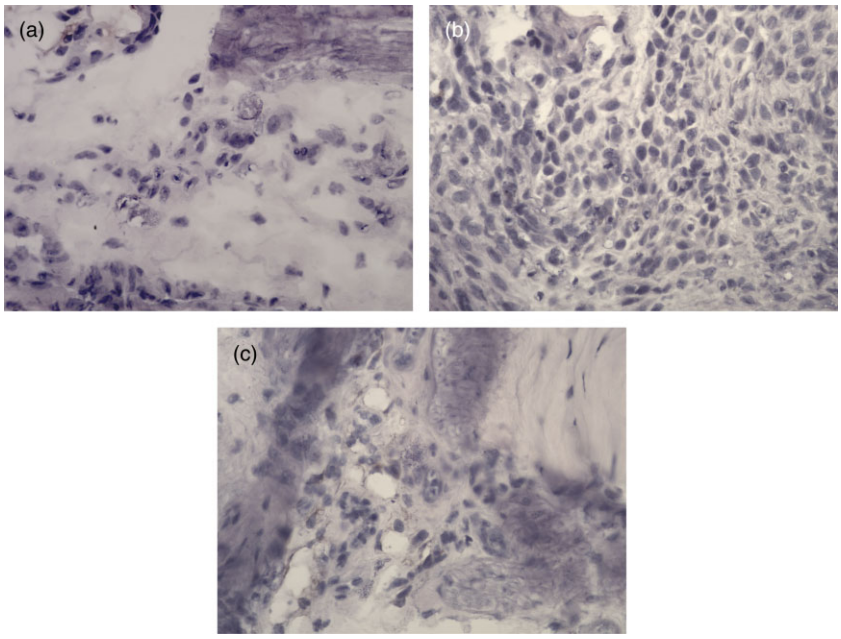

Fig. 2. Immune cell infiltration in the synovium. Representative images of the synovial infiltrate in hind paw joints of healthy controls (HC) (a), untreated collagen-induced arthritis (CIA) (b), and GN8P-treated CIA (c). Inflammatory infiltrate of the synovial and perisynovial tissue was stained by haematoxylin. Magnification ×40.

markedly down-modulated (Fig. 5c). Following the adaptive immune response, GN8P suppressed the percentage of both total and CD86-positive B lymphocytes (Fig. 5d,e). Moreover, we detected a substantial decrease of total T cells and both cytotoxic (Tc) and helper (Th) subsets (Table 1).

Cytokine serum levels, mRNA expression and anti-CII antibodies

The marked increase of tumour necrosis factor (TNF)-α caused by CIA was effectively prevented by the GN8P treatment (Fig. 6a). A similar observation was made with IFN-γ, where GN8P treatment kept the cytokine at the HC level (Fig. 6b). No significant changes were observed in the measured serum levels of IL-2, IL-4, IL-6 and IL-10. IL-17A showed a decreasing, although non-significant, trend after GN8P treatment (data not shown). As no significant changes of anti-inflammatory cytokines (IL-4, IL-10) were found in the sera, the mRNA expression was measured by real-time RT–PCR in SMC. While IL-10 expression was not altered by the GN8P treatment, IL-4 expression was markedly up-regulated (Fig. 6c).

The Th1 (IFN-γ)-driven IgG2a level was measured by ELISA. The results depicted in Fig. 6d demonstrate a

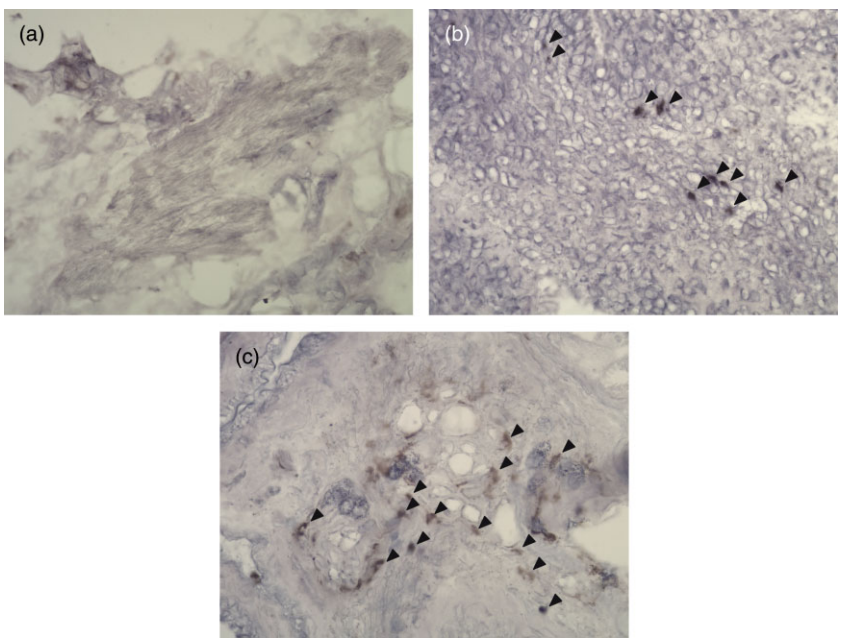

Fig. 3. Immunohistochemistry of NKG2D$^+$ cell infiltration in the synovium. Representative images of the synovial infiltrate in hind paw joints of healthy controls (HC) (a), untreated collagen-induced arthritis (CIA) (b), and GN8P-treated CIA (c). NKG2D-positive cells in the synovial and perisynovial tissue were stained by anti-NKG2D antibody. NKG2D-positive cells are marked with arrows. Counterstained with haematoxylin. Magnification ×40.

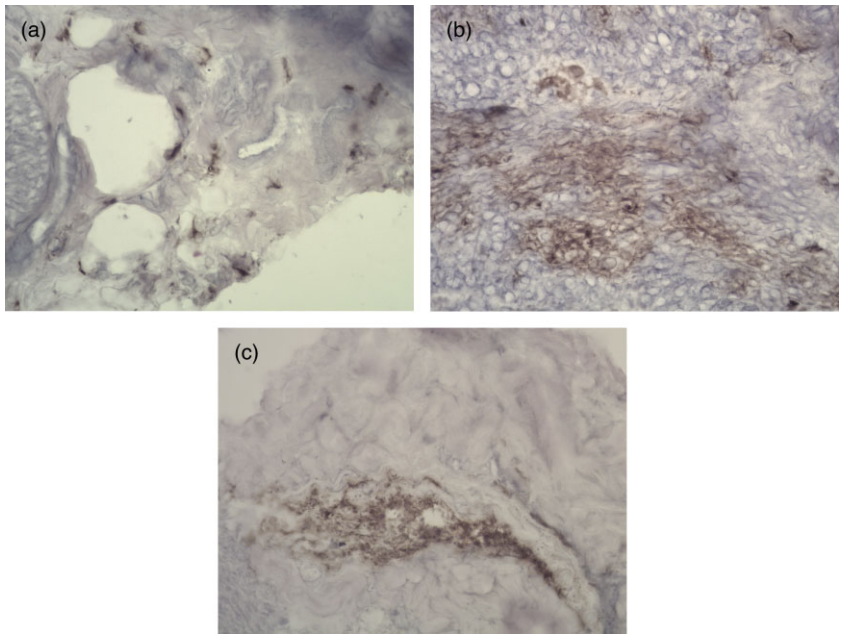

Fig. 4. Immunohistochemistry of CD11b$^+$ infiltration in the synovium. Representative images of the synovial infiltrate in hind paw joints of healthy controls (HC) (a), untreated collagen-induced arthritis (CIA) (b) and GN8P-treated CIA (c). CD11b-positive cells in the synovial and perisynovial tissue were stained by anti-CD11b antibody. Counterstained with haematoxylin. Magnification ×40.

14-fold increase of CII-specific antibody titre in CIA group relative to healthy controls. GN8P treatment prevented the rise of IgG2a by 7%.

Innate and adaptive immune responses to GCs

Due to the promising results obtained by GN8P administration to CIA mice, we further compared the effects of GN8P with another GlcNAc terminated conjugate on calix[4]arene scaffold – GN4C – providing better 3D structural control. We examined their effect on B cells, NK cells and antigen-presenting cells in the peripheral blood, spleen and lymph nodes and the effect on CIA clinical outcome.

We found a robust (fivefold) increase in total B cell percentage in the lymph nodes of CIA mice that was not changed by the GC treatment (Fig. 7a). Both GN8P and

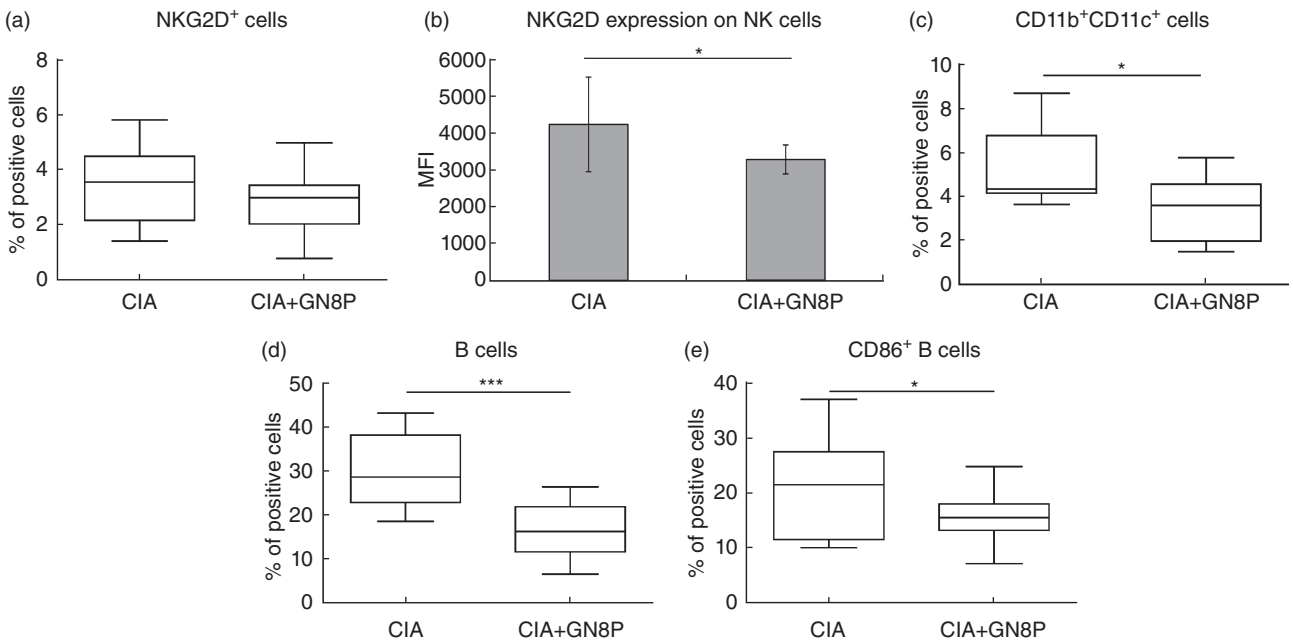

Fig. 5. Cytometric (FACS) analysis of synovial fluid. NKG2D-expressing cells in the synovial fluid (a), NKG2D expression [mean fluorescence intensity (MFI)] on NK cells (mean ± standard deviation) (b), relative distribution of CD11b$^+$CD11c$^+$ antigen-presenting cells (APCs) (c), CD45R/B220$^+$ B cells (d) and activated CD86$^+$ antigen-presenting B cells (e) in the synovia were measured by flow cytometry ($n = 5$–10 mice per group, $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$). Box-plots represent median, 25% and 75% percentiles, minimum and maximum.

Table 1. T cells distribution in the joints.

Subset	CIA	CIA+GN8P	*P*
T ($CD3^+NKp46^-$) (%)	3·6 ± 1·6	2·5 ± 0·7	<0·05
Tc ($CD3^+NKp46^-/CD8^+$) (%)	11·6 ± 2·1	6·1 ± 3·3	<0·01
Th ($CD3^+NKp46^-/CD4^+$) (%)	61·9 ± 5·9	52·9 ± 9·3	<0·05

The table shows the relative levels of total T, cytotoxic (Tc) and helper (Th) cells in the synovia as measured by flow cytometry. Data are represented as mean ± standard deviation. CIA = collagen-induced arthritis.

GN4C, however, prevented the activation of antigen-presenting ($CD86^+IA\text{-}IE^+$) B cells (Fig. 7b), as well as the generation of monocyte-derived ($CD11b^+CD11c^+$) antigen-presenting cells (Fig. 7c).

NK cell-mediated cytotoxicity was measured at three time-points during CIA development and compared to HC. SMCs from CIA mice on day 10 (the peak of CII+CFA-induced inflammation) displayed significantly higher cytotoxicity. On day 37 it was comparable to controls, whereas on day 50 it was decreased by 40%. GC administration did not influence the NK cell function in either of the administration schedules (Fig. 8a).

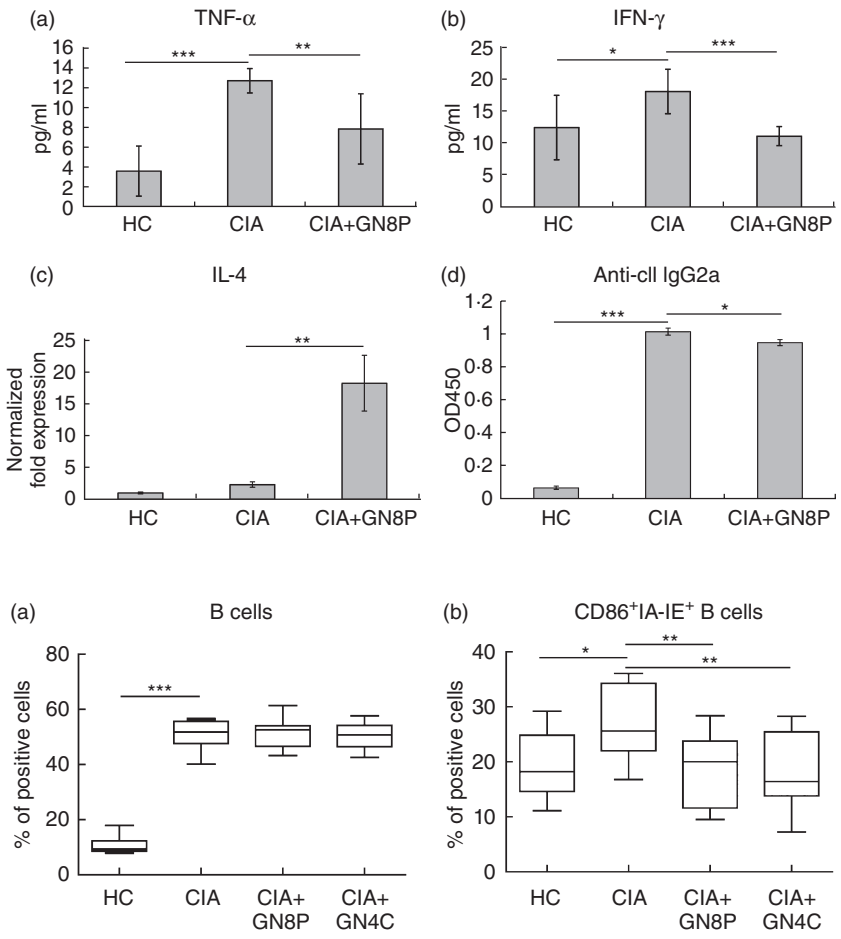

Fig. 6. Serum levels of TNF-α and IFN-γ, mRNA expression of IL-4 in spleen mononuclear cells (SMC) and type II collagen (CII)-specific IgG2a level. The inflammatory cytokines TNF-α (a) and IFN-γ (b) were measured in sera of healthy and collagen-induced arthritis (CIA) mice by cytometric bead array. Relative fold expression of IL-4 mRNA (c) was measured in SMC by real-time RT–PCR and normalized to β2-microglobulin [healthy controls (HC) = 1)]. Levels of CII-specific IgG2a (d) were determined by ELISA (n = 5–10 mice per group, sample dilution ×100). Data are expressed as mean ± standard deviation. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

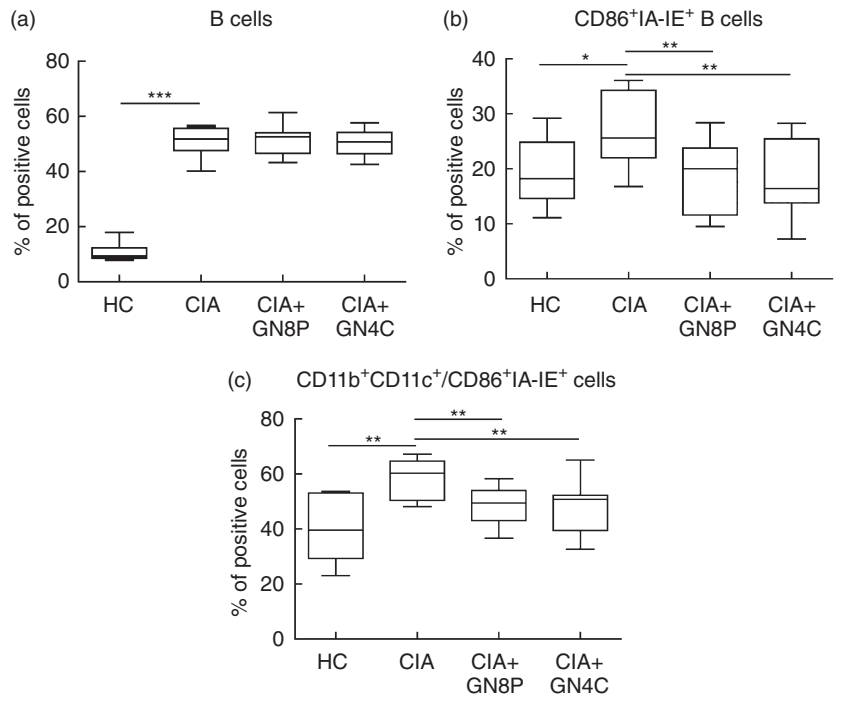

Fig. 7. Glycoconjugate-induced changes of relevant cell populations in the lymph nodes. Cells were isolated from the lymph nodes and analysed by flow cytometry (FACS). The relative distribution of $CD45R/B220^+$ B cells (a), activated $CD86^+$ antigen-presenting B cells (b) and myeloid $CD11b^+CD11c^+$ antigen-presenting cells (APCs) (c) are shown. Data represent results of three experiments performed (n = 5–10 mice per group) $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. Box -plots represent median, 25% and 75% percentiles, minimum and maximum.

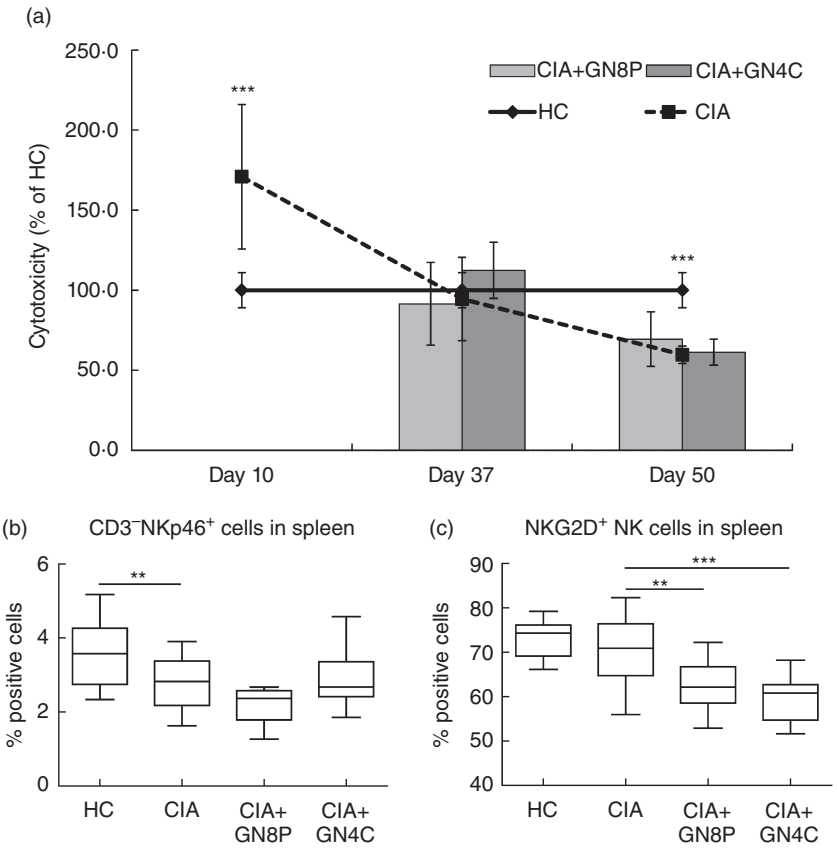

Fig. 8. Function, distribution and phenotype of splenic NK cells. Gradual impairment of splenic NK cytotoxicity during collagen-induced arthritis (CIA) development expressed as a percentage of NK cell-mediated cytotoxicity of healthy mice stated as 100% (specific cytotoxicity = 46·8 ± 5·1%) and the effect of presymptomatic and symptomatic glycoconjugate (GC) treatment (a). Effect of CIA and GC treatment on the relative distribution of NK cells in the spleen (b) and the subset of NKG2D$^+$ NK cells on day 37 (c). Results of three experiments performed (n = 5–10 mice per group) $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. Box-plots represent median, 25% and 75% percentiles, minimum and maximum.

With regard to the GCs' protective effect on NKG2D$^+$ infiltration into the synovia, we also evaluated the relative distribution of NK cells along with the expression of NKG2D in the spleen, blood and lymph nodes. CIA caused a drop of splenic NK cell proportion on day 37 that was not modulated by the GC treatment (Fig. 8b); however, NKG2D-expressing NK cell levels, not altered by CIA itself, were decreased significantly by both GN8P and GN4C treatments by 11 and 15%, respectively (Fig. 8c).

In the peripheral blood, the NK cell levels were not altered; however, CIA caused a decrease of NKG2D-expressing subset (HC = 75·8 ± 3·0%; CIA = 65·2 ± 5·3%; $P < 0{\cdot}01$), further slightly down-modulated by the GC treatment (CIA+GN8P: = 64·9 ± 5·9%; CIA+GN4C = 59·2 ± 7·6%). No changes in NK cell numbers and phenotype were found in the draining lymph nodes.

Amelioration of CIA clinical signs by the GC treatment

In our study, the CIA model evoked arthritic symptoms in 100% of untreated CII-immunized animals with the clinical onset on day 24·4 ± 2·5. The mean clinical onset of CIA was postponed significantly in GN4C-treated and non-significantly in the GN8P-treated mice (Fig. 9a). Concurrently, the GN4C and GN8P treatment caused a drop of incidence to 69 and 82%, respectively (Fig. 9b). The clinical symptoms and their severity, determined as mean score per affected limb, were attenuated significantly in GN4C-treated CIA mice (Fig. 9c). GN8P treatment resulted in a disease-moderating trend, reaching statistical significance in the final evaluated interval (day 32–37; $P < 0{\cdot}05$). Examination of the percentage of affected limbs per group revealed that GN8P caused a decrease of 17%, while GN4C caused a drop of 35% to half the level of untreated CIA controls (65%) (Fig. 9d).

Discussion

In this study we showed that the GlcNAc conjugates ameliorate the rheumatic symptoms of CIA, corresponding with the observations of Azuma *et al.* describing a similar effect of GlcNAc treatment in the SKG mouse model of RA [3]. GN8P prevented an inflammatory response in the arthritic joints by moderation of the immune cell infiltration and a significant reduction of both monocyte- and B cell-derived antigen-presenting T cells, and NKG2D-expressing NK cells in the synovial fluid. We found a GC-induced decrease of NKG2D$^+$ NK subpopulation in the spleen and both antigen-presenting cell types in the lymph nodes. GN8P administration prevented the rise of anti-CII IgG2a, IFN-γ and TNF-α level and up-regulated IL-4 mRNA expression in SMC, supporting the anti-inflammatory action of GlcNAc-conjugates. The beneficial effect of glucosamine or N-acetyl-glucosamine on the

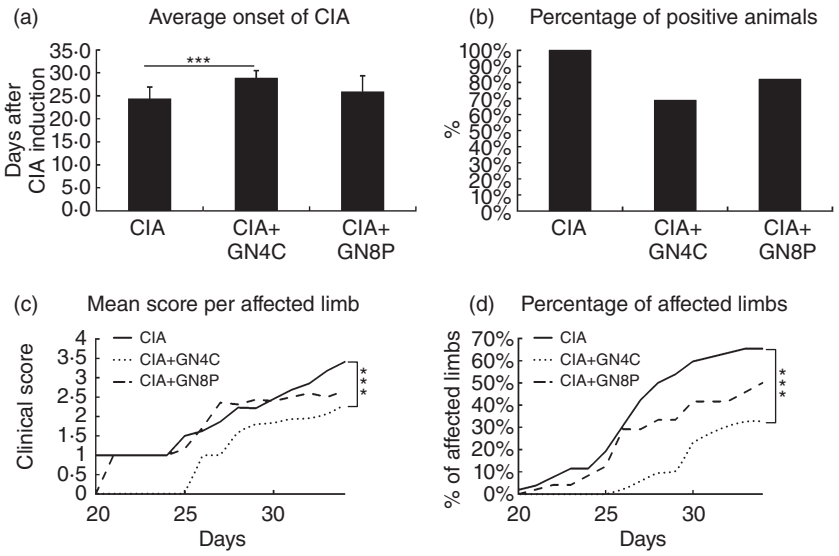

Fig. 9. Effects of glycoconjugate treatment on disease onset, incidence and clinical symptoms. Average time of onset of the collagen-induced arthritis (CIA) symptoms in untreated (CIA), GN4C and GN8P-treated groups (a). The percentage of ill animals in each group at the end of the experiment (day 37) (b). The progression of the disease severity was evaluated by clinical scoring (mean score per CIA-affected limb – c) and by the percentage of affected limbs per experimental group (d). Data represent results of three experiments performed ($n = 5$–10 mice per group), $^{***}P < 0{\cdot}001$.

disease progression and severity described previously in rodent arthritic models [3,9] was thus supported by our results using multivalent GlcNAc-terminated conjugates.

The histological evaluation of the CIA-affected joints showed severe cell infiltration (comparable to the results of Joosten *et al.* [27]) and particularly the presence of NKG2D and CD11b-positive cells, reported previously to participate in bone erosion [28,29]. The presymptomatic glycoconjugate treatment protected from the $CD11b^+$ (notably the $CD11b^+CD11c^+$ antigen-presenting cells), B and T cell infiltration. Although the mechanism is unknown, Ma *et al.* demonstrated analogous suppression of dendritic cells (DC) and T lymphoblast activation *in vitro* by glucosamine [30]. These effects, together with the down-modulation of NKG2D expression by NK cells in the joints, could participate in the beneficial clinical outcome of the GC treatment, comparable to blocking of NKG2D receptor by antibodies demonstrated by others [31,32].

In our presymptomatic treatment schedule, GN8P lowered the $NKG2D^+$ NK cell percentage; however, GN4C was more effective, which corresponded with the attenuation of clinical symptoms. Unlike Andersson *et al.*, who affected cells at the site of ongoing inflammation by anti-NKG2D treatment [32], we also managed to down-modulate NKG2D-positive cells at the systemic level. We consider these findings important with regard to the negative effect of NKG2D on autoimmunity development reported previously [31,33]. Evaluating NK cytotoxicity, however, we also detected the suppression of NK effector function appearing during the disease progression, as reported by Aramaki *et al.* in human RA [21] or Lo *et al.* in CIA [22]. During the early arthritis phase the cytotoxicity was at the HC level, while in the late arthritis phase it showed a highly significant suppression. The lower relative distribution of NK cells in the spleen on day 37 in CIA-suffering mice also corresponded with this effect. A robust increase before the onset of clinical symptoms can be attributed to the stimulation by CFA [34]. With regard to the effect of our treatment, we incline to the concept that NK cells rather play a proinflammatory role in RA, as presented by de Matos *et al.*, [19], Dalbeth *et al.* [20] or Pridgeon *et al.* [35], and their suppression is beneficial for the clinical outcome. The GC treatment leading to down-modulation of NKG2D on NK cells, which results in amelioration of the disease outcome, although in a manner yet unknown, merits further investigation.

As multi-competent lymphocytes with the ability to regulate innate and adaptive immune responses, NK cells have been shown to determine the outcome of B cell-mediated autoimmunity [36] and antigen-presenting cell activation [37]. B cells were also proved to be the key players in CIA development [38]. We saw a massive (five-fold) increase of B cells in the draining lymph nodes of CIA mice, confirming their activation and proliferation, corresponding with the subsequent CII-specific IgG2a antibody formation, partially prevented by the GN8P treatment. IgG2a is a Th1-driven isotype induced by IFN-γ [39], which is a prominent product of NK cells. Williams *et al.* [40] also described a correlation between antibody titres and disease severity in CIA, which corresponds with our results demonstrating the attenuation of the clinical symptoms, although the clinical improvement cannot be attributed to this phenomenon only.

In the synovia we observed a GN8P-caused decrease of total B cells and CD86-expressing B cell subset. The CD86-positive B cells are critical for B–T cell communication, and have an essential role in the activation of autoreactive T cells in RA [41]. Thus, the GC treatment, beside the reduction of antibody formation, inhibits the infiltration of autoreactive T cells into the synovia. This fact corresponds with the decreased numbers of T cells (particularly Tc) found in the joints.

Other important players in both human RA and animal models are the proinflammatory cytokines [42,43]. TNF-α participates in the pathogenesis of RA as a paracrine stimulator of IL-1 and GM-CSF [44,45] and drives the synovial inflammation leading to joint destruction [46]. GN8P prevented the CIA-evoked TNF-α production together with a drop of $CD11b^+CD11c^+$ synovia infiltration, resembling the favourable clinical effect caused by direct blockage of TNF-α [47]. Similar results were achieved by anti-TNF therapy of RA patients decreasing the CD86-expressing B cells [48]. IFN-γ contributes to arthritic inflammation by the activation of macrophages and IgG2a isotype switch [39,49]. GC application, starting at the time of peak inflammatory reaction, when it can affect the processes leading to the disease establishment, can successfully prevent the IFN-γ production leading to partial suppression of the anti-CII IgG2a production and antigen-presenting cell activation.

It has been shown that IL-4 is a crucial anti-inflammatory mediator of CIA, correlating with tolerance induction. CIA can be suppressed by intravenous injection of CII; however, in DBA/1 $IL4^{-/-}$ mice, such treatment is completely ineffective [50]. IL-4 also potentiates CIA treatment by dexamethasone [51]. We thus examined the mRNA expression of IL-4 in splenocytes, although its serum level was not altered significantly. GN8P treatment caused a major increase in IL-4 mRNA expression, which supports the notion that it functions as an important suppressor of CIA development.

Grigorian *et al*. demonstrated a relationship between the inhibition of Th1 and Th17 cytokines and disease progression in experimental autoimmune encephalomyelitis (EAE) after oral administration of GlcNAc [52]. We assume a similar, although yet not fully unravelled, effect after the GlcNAc glycoconjugate treatment of CIA, orchestrating the complex interaction of both the innate and adaptive immune responses. We suggest that GCs influences directly, or via the cytokine network, antigen recognition and intercellular communication between antigen-presenting cells, NK and activated $CD86^+$ B cells, resulting in down-modulation of Th1 and autoaggressive $CD8^+$ T cells. Moreover, we assume a possible influence of IL-4 that would be effective in suppressing the autoimmune reaction, by induction of $CD4^+CD25^+$ regulatory T cells, as reported by Skapenko *et al*. [53,54].

Taken together, the prophylactic GC treatment is capable of effective reduction of the incidence and postponement of the CIA onset as well as moderation of its severity. This is caused particularly by the prevention of inflammatory infiltration, inhibition of antigen presentation by both B cells and professional antigen-presenting cells and suppression of proinflammatory cytokine production. We suggest that inhibition of NKG2D-positive NK cells also plays an important role here, leading to the moderation of autoimmune processes. Our results prove that the glycobiological aspect should not be overlooked, as it may bring exciting new information and provide new prospects for more effective therapeutic interventions.

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Disclosure

The authors declare no conflicts of interest.

Author contributions

J. R., K. C., V. H., I. D., L. V. and A. F. performed the experiments; J. R. and A. F. designed the study; M. M. performed the statistical evaluation; J. R., M. M. and A. F. wrote the manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Glycoconjugate administration schedule. Synthetic glycoconjugates were administered in two different administration schemes – presymptomatic (a) and symptomatic (b). The average onset of clinical symptoms was at day $24 \pm 2{\cdot}5$ after immunization by type II collagen + complete Freund's adjuvant (CII+CFA). In the presymptomatic scheme, glycoconjugates (GCs) were administered at days 10, 13, 16, 23 and 30. In the symptomatic scheme, GCs were administered in the same time intervals starting from day 23 (first appearance of symptoms). Animals were killed at days 37 and 50 in presymptomatic and symptomatic schedules, respectively (in both cases 7 days after the last treatment dose).

4. Discussion

The goal of this thesis was to shed some light on the complex processes leading to rheumatoid arthritis with special regard to innate immune response and its modulation with synthetic, multivalent glycans. Special attention was paid to the role of NK cells, their functional properties and receptor repertoire. The research started with an extensive study on human RA-suffering patients where we concentrated on the role of NK cells in the disease and the possible modulation of their function by GN8P glycoconjugate. Aberrant glycosylation present in the rheumatic conditions is being studied as an important factor playing role in the regulation of autoimmunity [215], although the exact mechanisms remain mostly unknown. We focused on NK cell function in blood of the RA-suffering patients and found its significant suppression. These results correlated with the phenotype of NK cells expressing varying levels of CD161 inhibitory receptor and with the reaction to the synthetic glycodendrimer added *in vitro*. At the time of the project development and execution it was believed, that GN8P is an established synthetic ligand of CD161 [216], so correlation of the NK cell function in the presence of GN8P with the expression of CD161 (NKR-P1A) was a valid argument. This hypothesis was, however, disproved recently [217], so with regard to these findings, we cannot attribute the GN8P-caused inhibition unambiguously to its binding to CD161 receptor, although the reaction of the cells to the glycoconjugate closely correlates with the intensity of its expression. It is also important to note that LLT1 – a natural ligand of CD161 showed similar action [177]. The exact way of GN8P interaction with NK cells is currently a question of further research, although its effect on NK cell function is undisputed.

During the study, we evaluated MCV as a known autoantigen in RA and tested its effect on NK cells and their phenotype [218]. We found important differences between patients and healthy donors in reaction to MCV and expression of CD161. It is highly probable that MCV plays its role in NK cell inhibition. We also found elevated expression of PAD4 in reaction to MCV in healthy donors. This enzyme has multiple associations with inflammation. It produces citrullinated antigens such as MCV that induce the formation of ACPA (anti-citrullinated protein antibodies) immune complexes and stimulate TNF-α production, leading to amplification of the autoinflammatory reaction. On the other hand, TNF-α has been shown to reciprocally induce PAD4 activity [219]. This result suggests that NK cells, that were also previously proved to express PAD4 [220], may also play its role in the citrullination process taking place during RA development and progression, however, the number of papers is rather limited and this topic requires further investigation.

As RA was proved to be related to dysregulation of glycosylation processes [179], we studied MGAT5 glycosyltransferase required for building N-glycan complexes. For the first time we showed that MGAT5 is active in the synoviocytes of the patients, suggesting that the N-glycan structures present in the inflamed synovium may have inhibitory effect on NK cells, although probably indirect. More detailed analysis

of the expression and function of MGAT3 and MGAT5 glycosyltransferases in NK cells was also the topic of the second presented paper. GN4C – another, more rigid, GlcNAc glycodendrimer was used here as a prototype structure for studying NK cell reaction to glycans. Here we used fresh human NK cells and NK-92 cell line to be able to compare the effects *in vitro*. The use of purified NK cells either from the cell line or from healthy donors in the *in vitro* system shows the direct effect of the treatment by the glycodendrimer as there are no other cell types that could mediate any indirect effects. The prominent result was the stimulation of IL-2 production in both NK-92 and fresh NK cells. IL-2 is a potent autocrine stimulator not only in T cells [221, 222] but also in NK [197, 223]. Such stimulation is particularly important in antitumour immune response where NK cells form the first line of defence (reviewed e.g. in Zamai et al. [224]). In both models, we also showed that GN4C downmodulated the expression of both glycosyltransferases, interfering with the glycosylation processes.

Monitoring NK cytotoxicity, we found indirect correlation between CD161 expression and cytotoxicity level in human RA PBMC samples; however, we did not monitor the expression after *in vitro* treatment with the glycoconjugate. In the second paper, we tested the *in vitro* reaction to GN4C on purified NK cells and found its suppressive effect on NKR-P1A expression. Further, we found upregulation of cytotoxicity as well as an increase of NKG2D expression. As the previous study showed NK function impairment after GN8P treatment, it is necessary to view the results in a wider context. As it is improbable that the similar dendrimers would have such different effect, we can attribute the discrepancy to the different model. Not concerning NK-92 cell line as a rather artificial tumour model, the main difference between the two studies lies in the use of PBMC in case of the first study and FACS-sorted NK cells of healthy donors used in the other one. As GlcNAc was reported to affect a number of other cell types, this would suggest an important role of cellular co-operation in case of tests on PBMC, forming a more complex milieu of mutual interactions, where the effect on the NK cell function is just one of its manifestations. One of the possible explanations is the inhibitory effect of GlcNAc on Th1 and Th17 cells reported by Grigorian et al. [175], leading to NK cells functional suppression via lack of IL-17 stimulation [225].

In the last study, we used both previously mentioned glycodendrimers as modalities for treating collagen-induced arthritis in mice. We were led by the fact that GlcNAc is being used as a well-tolerated supplement for the treatment of osteoarthritis [201, 226]. Beneficial effects of GlcNAc on rodent arthritis models were also shown by Azuma et al. and Hua et al. [200, 202]. Knowing the immunomodulatory capabilities of the glycodendrimers described in the previous two papers as well as other studies [194, 195, 203], we were interested in their possible use as treatment agents.

We used the well-established CIA model in DBA/1 mice, particularly because other models such as CAIA [53] do not sufficiently simulate the situation appearing at the initial stages of autoimmunity development. During the study, we tested two administration schemes of the glycoconjugates – presymptomatic attitude showing promising results and symptomatic treatment that did not show such strong effects.

Although the symptomatic schedule better resembles the therapeutic approach to human patients, even the presymptomatic schedule cannot be considered purely preventive because, although it was started before the clinical manifestation of the disease, the processes leading to CIA had already been initiated by CII injection. This is an important fact, showing the possible use of (multivalent) GlcNAc in the treatment of early arthritis.

Inflammatory infiltration by both myeloid and lymphoid cells in the joint is, as well as cartilage erosion, the hallmark of not only CIA but also RA [227-229]. Our treatment effectively inhibited the infiltration by CD11b-positive cells, B cells and T cells that all contribute to the ongoing inflammatory processes. Analogous immunosuppressive effects of glucosamine were previously shown *in vitro* by Ma et al. [187]. Reproducing similar reaction in situ is particularly important as it directly leads to attenuation of the arthritic symptoms.

NKG2D was previously reported to have negative effects during the progression of autoimmunity [127, 230], so we monitored the percentage of NKG2D-expressing cells at the site of inflammation as well as in the spleen. Our results showing the reduction of both NKG2D-positive cell percentage and its expression can thus be considered rather positive. It is, however, necessary to remark that the suppression of NKG2D expression by GC treatment *in vivo* does not correspond with the effects observed after GN4C treatment *in vitro* as reported in publication 2 [205].

Seeing these results in wider context suggests the involvement of a more complex network of cellular interactions taking part in the reaction to glycans *in vivo*. Cytokines, that have wide potential to modulate immune responses, were in this regard particularly interesting for us. We concentrated mainly on TNF-α and IFN-γ as two pro-inflammatory cytokines playing important roles in the pathogenesis of RA. TNF-α drives the synovial inflammation resulting in joint destruction as well as it stimulates the production of other pro-inflammatory agents (IL-1 and GM-CSF) [28, 231, 232] and therefore represents one of the targets of modern therapy by "biological response modifiers" (reviewed by Geiler et al. [233]). IFN-γ is, on the other hand, important for stimulation of antibody response, pushing antibody class switch towards the production of IgG2a [234]. The application of GCs caused an important prevention of the rise of both cytokines, corresponding with the attenuation of clinical symptoms, lowering the CII-specific IgG2a but also with the decrease of CD86-expressing antigen-presenting B cells. This B cell subset is particularly important in the development of RA [208] and represents one of the targets of anti-TNF-α therapy (Adalimumab) used in patients [213]. IL-4 was measured as an essential anti-inflammatory mediator of CIA [235]. The increase in its expression caused by the GC treatment, corresponded with the reduction of IFN-γ and inflammatory Th1 response, and thus led to the suppression of CIA development.

One of the reasons for the study on CIA mice was to compare the alterations of NK cell function found in human patients with the animal model. In RA patients, we found a marked suppression of NK cell function that could be modulated by GN8P *in vitro*. In CIA mice, we performed a series of cytotoxicity assays to prove the NK cell suppression showed by Lo et al. [160], presenting the progressive NK cell suppression in time. We found gradual decrease of NK cell function in CIA mice, resembling the situation in RA patients, particularly after the appearance of the symptoms. This finding proves that CIA is a valid animal model of RA in the context of NK cell function and the development of the disease and it is also interesting to note that while there were PBMC samples used in the RA study, in CIA mice, we used splenocytes with similar outcome. Using the animal model of RA, we were able to test the *in vivo* effect of the glycodendrimers. Although they had several important, positive effects described above, there was no detectable influence on either the function of NK cells, or their percentage. Nevertheless, there was a significant effect on the activation status of the NK cell population showed by the number of NKG2D-expressing cells. In conclusion, our work showed that the GC treatment of CIA mice does not influence only NK cells but that there is a number of other cell types involved, including APCs or B cells as well as the cytokine network. The effect on NK cell function *in vivo* is thus likely to be mediated by a number of indirect reactions.

5. Conclusions

In this work, we studied the role of NK cells and the immunomodulation capacity of multivalent glycans in three different models. In human RA we focused on the description of NK cell involvement in the pathological processes leading to the disease with special regard to the role of glycans and RA-specific autoantigen. Studying isolated NK cells of healthy donors *in vitro* provided us with a more detailed insight into the molecular processes taking place in reaction to the presence of GlcNAc-carrying structures. Finally, the use of CIA as a complex and relevant mouse model of human RA enabled us to evaluate the treatment potential of GlcNAc dendrimers *in vivo*, under autoimmune conditions well comparable to RA patients.

The work provided here brought the following important conclusions:

- **NK cells respond to MCV – the RA-specific autoantigen – that may serve as a triggering mechanism of CD161 expression correlating with NK cell functional impairment observed in RA patients. Although the exact mechanism is yet unknown, cells of patients and healthy donors with higher expression of CD161 react to GlcNAc glycoconjugate by inhibition of cytotoxic function, supporting the role of glycosylation in the rheumatic process. Human NK cells were also found to express PAD4 citrullination enzyme, and may thus participate in the citrullination processes taking part in RA pathogenesis.**
- ***In vitro* treatment with the glycoconjugates inhibits MGAT3 and MGAT5 glycosyltransferase expression in purified NK cells, as well as in synovial cells of RA patient joints, interfering with glycosylation pathways.**
- **The glycoconjugate treatment of CIA mice *in vivo* inhibits inflammatory infiltration of the joints and suppresses NKG2D expression, pro-inflammatory cytokine production, and antigen presentation. These immunomodulating effects result in postponement of the disease onset and amelioration of pathological symptoms, which is beneficial for the clinical outcome.**

Taken together, this work brings a comprehensive overview of NK cell involvement and glycan functioning under different *in vitro* and *in vivo* conditions in health and disease, bringing new insights into the immunopathogenesis of rheumatoid arthritis and suggesting potential new prospects for more effective therapeutic interventions.

6. References

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Seznam publikací / List of publications:

Publikace, které jsou podkladem disertace / Publications included in the thesis

- **Richter J**, Benson V, Grobárová V, Svoboda J, Vencovský J, Svobodová R, Fišerová A. CD161 receptor participates in both impairing NK cell cytotoxicity and the response to glycans and vimentin in patients with rheumatoid arthritis. Clinical Immunology 2010;136:139-47. **IF=3.93**
- Benson V, Grobárová V, **Richter J**, Fišerová A. Glycosylation regulates NK cell-mediated effector function through PI3K pathway. International Immunology 2010;22:167-77. **IF=3.30**
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Publikace bez vztahu k tématu disertace / Publications not included in the thesis

- Větvička V, Dvořák B, Větvičková J, **Richter J**, Křížan J, Šíma P, Yvin JC. Orally administered marine (1-3)-beta-D-glucan Phycarine stimulates both humoral and cellular immunity. International Journal of Biological Macromolecules 2007; 40: 291-8. **IF=1.58**
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2001–2006 – Univerzita Karlova v Praze, Přírodovědecká fakulta, Magisterské studium, obor – biologie, specializace – imunologie.
Diplomová práce / Diploma thesis: Studium vlivu β-1,3-glukanu aplikovaného v dlouhodobém pitném programu na vybrané imunitní parametry

1995–2001 – Gymnázium Botičská, Praha 1 – přírodovědné zaměření

Členství v odborných společnostech / Memberships:

Česká imunologická společnost (Czech Immunological Society)
Society for Natural Immunity, USA

Kurzy a workshopy / Courses and workshops:

2014 – Osvědčení o odborné způsobilosti k navrhování pokusů a projektů pokusů podle § 15d odst. 3 zákona č. 246/1992 Sb., na ochranu zvířat proti týrání, ve znění pozdějších předpisů (ev. č. CZ 01834)
2012 – FP7 DINAMO Workshop 2012 – nanotechnologie v biomedicíně
2009 – Kurz digitální průtokové cytometrie
2008 – Kurz fluorescenční mikroskopie a mikroskopické imunodetekce v biomedicíně
2008 – Osvědčení o způsobilosti podle §17 zákona ČNR č. 246/1992 Sb., na ochranu zvířat proti týrání v platném znění (ev. č. ČZU 1203/08).

Konference / Conferences:

E-MRS Spring meeting, 2012, Strasbourg, Francie
Přednáška / Lecture:
Richter J, Petráková V, Cígler P, Ledvina M, Nesládek M, Kreuger A, Fišerová A: Detection and targeting of fluorescent nanodiamonds in biological samples.

SNI meeting 2012 - NK2012, 2012, Pacific Grove, USA
Posterové sdělení / Poster presentation:
Richter J, Čapková K, Benson V, Burócziová M, Vannucci L, Fišerová A: GlcNAc Glycocojnugate treatment affects NK cell regulátory function in mouse modelof rheumatoid arthritis (CIA).

SBDD XVII, 2012, Hasselt, Belgie
Přednáška / Lecture:
Richter J, Werner L, Petráková V, Míčová J, Meinhardt T, Krueger A, Flegel M, Cígler P, Fišerová A, Štursa J, Ledvina M, Nesládek M: Preparation of selectively targeted fluorescent nanodiamond as universal tool for biomolecuar delivery and detection in cancer cells.

Cancer Conference 2011, 2011, Xcaret, Mexiko
Posterové sdělení / Poster presentation:
Richter J, Petráková V, Cígler P, Ledvina M, Grobárová V, Čapková K, Nesládek M, Fišerová A: Fluorescent nanodiamonds as non-toxic probes for monitoring of cancer processes.

Konference ČSAKI 2011, 2011, Košice, Slovensko
Posterové sdělení / Poster presentation:
Richter J, Cígler P, Ledvina M, Petráková V, Čapková K, Fišerová A: Fluorescent nanodiamonds as a detection and delivery system for cancer monitoring.

First Tutorial Workshop in Immunology, 2010, Praha
Posterové sdělení / Poster presentation:
Richter J, Benson V, Grobárová V, Svoboda J, Vencovský J, Svobodová R, Fišerová A: CD161 is responsible for impaired NK cell cytotoxicity and response to glycans and vimentin in patients with rheumatic diseases.

12th Meeting of the Society for Natural Immunity & NK2010, 2010 Dubrovnik, Chorvatsko
Posterové sdělení / Poster presentation:
Richter J, Benson V, Grobárová V, Tichá V, Kovářů H, Havrdová E, Vencovský J, Fišerová A: Participation of C-type lectin receptors in the pathogenesis of autoimmune diseases.

World Immune Regulation Meeting, 2010, Davos, Švýcarsko
Posterové sdělení / Poster presentation:
Richter J, Benson V, Grobárová V, Svoboda J, Vencovský J, Svobodová R, Fišerová: CD161 is responsible for impaired NK cell cytotoxicity and response to glycans and vimentin in patients with rheumatic diseases .

30th Congress of the Société Internationale d'Urologie, 2009, Shanghai, Čína
Posterové sdělení / Poster presentation:
Richter J, Vinakurau S, Svoboda J, Cimburek Z, Rosina J Fišerová A: Advanced flow cytometry for evaluation of stress protein recognition by CD161 and NKG2D in the course of radiation therapy in prostate cancer patients.

Konference ČSAKI 2008, 2008, Praha
Posterové sdělení / Poster presentation:
J. Richter, J. Svoboda, Z. Cimburek, H. Kovářů, V. Tichá, R. Svobodová, E. Havrdová, A. Fišerová: Mnohobarevná průtoková cytometrie pokrývající buňky specifické i vrozené imunity s důrazem na CD161 umožňuje bližší poznání patogeneze RA a RS

6th International Congress on Autoimmunity, 2008, Porto, Portugalsko
Posterové sdělení / Poster presentation:
Richter J, Svoboda J, Cimburek Z, Kovářů H, Tichá V, Svobodová R, Havrdová E, Fišerová A: Advanced FACS analysis covering specific and innate immunity with emphasis on CD161 enables insight into pathogenesis of RA and MS.

ISAC conference, 2008, Budapest, Maďarsko
Posterové sdělení / Poster presentation:
Richter J, Svoboda J, Cimburek Z, Fišerová A: Multiparametric flow cytometry enables wide-range screening of patients suffering from autoimmunity in relevance to CD161 expression.

Kongres ČSAC, 2007, Brno
Posterové sdělení / Poster presentation:
Richter J, Cimburek Z, Svoboda J, Novotná L, Kuldová M, Růžičková Š, Fišerová A: FACS analysis of CD161 and NKG2D on various cell subsets in autoimmunity.